SPECIFICATION AND FUNCTIONAL SIGNIFICANCE OF THE LEAF ANATOMY OF C_4 PLANTS

by

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STATEMENT

This thesis describes my own original work.

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This thesis describes the results of research work carried out in the Taxonomy Unit and Department of Environmental Biology, Research School of Biological Sciences, Australian National University, during the tenure of an A.N.U. Postgraduate Scholarship.

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ABSTRACT

Leaf transections of over 300 plant species have been observed light microscopically with the aims of specifying leaf anatomical features associated with the C_4-dicarboxylic acid pathway of photosynthesis, and of relating these to C_4 plant biochemistry and physiology. The sample, mainly grasses, includes both C_4 and C_3 species, and representatives of most of the C_4 anatomical variants currently known.

For grasses, two parameters have been found which reliably distinguish C_3 from C_4 species. A "maximum lateral cell count" of 2-4, and a "maximum cells distant count" of 1, are characteristic of C_4 grass leaves, and appear to be of fundamental physiological significance. C_3 grass leaves have a maximum lateral cell count of >4, and a maximum cells distant count of >1.

The grass family also contains different biochemical C_4 type species, and these can be distinguished using the XyMS character. Primary vascular bundles of leaves of "NAD-Malic Enzyme" or "Phosphoenolpyruvate Carboxykinase (PCK) type" species are characterised by the presence of cells in the mestome sheath position between metaxylem vessels and laterally adjacent bundle sheath (= "Kranz" = "photosynthetic carbon reduction [PCR]") cells: i.e., they are XyMS+. "NADP-Malic Enzyme type" C_4 species lack such cells, and are classed as XyMS-. The XyMS character was used to predict C_4 type for over 250 grass species. The character may indicate that routes of photosynthate transport to phloem loading sites may differ between C_4 species types.

The existence of a variety of C_4 leaf anatomical types is not only of value in defining constant anatomical features of C_4 plant leaves. In the future, these variants should help in assessing the functional significance of intra-C_4 anatomical variation (e.g., the XyMS character). Identification of the two chlorenchymatous cell types in C_4 plant leaves is an essential starting point, and this was achieved for PCR cells by immunofluorescent labelling of ribulose-1,5-bisphosphate carboxylase (RuP_2Case) in PCR cell chloroplasts. The technique has been applied to leaves of over 40 species

(C_3, C_4, and one CAM species) from seven monocotyledonous and dicotyledonous families. It is especially useful for species with leaves of unusual (= "non-classical") C_4 anatomy, for hybrids (e.g., of *Atriplex*), and for putative C_3/C_4 intermediates (e.g., *Panicum milioides*). The result for *P. milioides*, considered in relation to other known experimentation on this species, has led to a hypothesis on the intermediate nature of its photosynthetic carbon metabolism. Immunofluorescent labelling of RuP_2Case supports previous evidence on the cellular location of this enzyme in species with normal (= "Kranz" = "classical") C_4 leaf anatomy.

I conclude that five anatomical features are characteristic of C_4 plant leaves (and these are discussed in relation to the biochemistry and physiology of C_4 photosynthesis); 1) that two chlorenchymatous cell types exist which perform the bicellularly compartmented biochemical events of C_4 photosynthesis: PCR cells, and "primary carbon-assimilation (PCA)" (= "mesophyll") cells; 2) that PCA and PCR tissues are usually juxtaposed, but if not, they are separated by cells usually $\leq 10\mu m$ in diameter; 3) that PCA cells are always found between PCR cells and the atmosphere; 4) that PCR-PCA and PCR-intercellular gas space contact is small: viz, PCR cells are arranged in some sort of layer on one side only of which do gas spaces in contact with the atmosphere occur (exceptions are isolated PCR strands); inter-PCR cell gas spaces do not exist; PCR cell surface area/volume ratio is low; 5) that PCA and PCR tissues are quantitatively balanced, and this mirrors the stoichiometry of bicellularly compartmented biochemical events in C_4 photosynthesis. This "anatomical stoichiometry" is reflected in such parameters as the maximum lateral cell count of 2-4, the maximum cells distant count of 1, low interveinal distance, low PCA/PCR numerical cell ratio, and low PCA/PCR volume ratio.

Preliminary data on PCA/PCR volume ratios are of interest with respect to different C_4 types also, NAD-ME and PCK type ratio ranges being lower than that for NADP-ME type species. Such ratios may be related to PCR cell shape, PCR cell radial dimensions, and/or different PCA/PCR tissue capacity requirements for the different C_4 species types.

Anatomical and preliminary experimental observations on leaf sheaths of over 50 C_3 and C_4 grass species support conclusions from leaf blade observations, although C_4 sheath anatomy usually deviates from the popular conception of C_4 leaf anatomy (viz. "Kranz" anatomy).

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CHAPTER 1

INTRODUCTION

1.1 INITIAL RESEARCH IDEAS

The work presented in this thesis had its origins in a written review in which I set out to integrate detailed information on the biochemistry and physiology of the C_4-dicarboxylic acid pathway of photosynthesis, photorespiration, anatomy and ultrastructure, carbon isotope discrimination, and grass systematics. I also considered briefly, such topics as C_3/C_4 intermediacy, C_3/C_4 hybrids, evolution of C_4 photosynthesis, photochemistry, and chlorophyll a/b ratios.

This rather exhaustive review, which occupied most of my time from November 1973 to late 1974, has now been extensively superseded, and many of the topics I considered have since been dealt with in symposia and reviews (Bielski *et al.* 1974; Björkman *et al.* 1974; Laetsch 1974; Avron 1975; Chollett and Ogren 1975; Marcelle 1975; Zelitch 1975; Burris and Black 1976; Hatch 1976a; Hatch and Osmond 1976; Kelly *et al.* 1976). In addition, I have accumulated more than 250 relevant papers, which have been published in the last two years.

Presentation of an updated version of my review has seemed superfluous. The aim in preparing the review, however, was to map out areas promising to repay further study, to ask pertinent questions, and to generate ideas which seemed worth testing. The questions and ideas, *as conceived in 1974*, are set out below. Some of them remain relatively unexplored, while others were partly pursued by me in this thesis as indicated by the Chapter references given.

(1) Can C_4 leaf anatomy be characterised precisely? Or, what are the functionally-essential anatomical features of C_4 plant leaves? Unusual C_4 leaf anatomical types (e.g., see Johnson and Brown 1973) might provide useful clues. (Chapters 2, 5, and 8).

(2) Are there any anatomical differences between C_4 species types (e.g., bundle sheath cell shape)? I had noted that in longitudinal

section, leaf blades of *Digitaria sanguinalis* exhibited bundle sheath cells elongated in the longitudinal axis of the leaf blade (illustrated Edwards and Black 1971a). From available information, it was reasonable to assume that this species was an NADP-malic enzyme species (= "malate former"). By contrast, paradermal sections of leaf blades of *Panicum miliaceum* ("aspartate former"; NAD-malic enzyme species), *Atriplex spongiosa* ("aspartate former"), and *Amaranthus edulis* ("aspartate former") (illustrated Hatch and Mau 1973) revealed square-shaped bundle sheath cells. In longitudinal section, *Panicum maximum* ("aspartate former"; PEP carboxykinase species) exhibited bundle sheath cells tending to be elongated radially to the vascular bundles (illustrated Hatch and Mau 1973). (Chapters 3 and 7).

(3) Can ribulose-1,5-bisphosphate carboxylase (RuP_2Case), and other enzymes, be localised in any other way than by using cell separation techniques? Several reports question the cellular compartmentation of enzymes such as RuP_2Case: viz. Baldry *et al.* 1971; Bucke and Long 1971; Bednarz and Rasmussen 1972; Poincelot 1972). Can Fraction I protein be observed in all grasses electron microscopically (as done, perhaps, in *Avena:* Gunning *et al.* 1968). (Chapters 4 and 5).

(4) Can the cellular compartmentation of photosynthetic enzymes be demonstrated in C_4 plants exhibiting unusual C_4 leaf anatomy: viz. *Aristida*, *Arundinella*, *Cyperus*, *Fimbristylis*, and *Triodia* types; e.g., using cell separation techniques? (Chapter 5).

(5) How do other green parts of a C_4 grass (e.g., leaf sheaths, stems, glumes, paleas, lemmas) compare with leaf blades anatomically, physiologically, and biochemically? (Chapter 6).

(6) Is the stoichiometry of the in-series biochemical events of the two chlorenchymatous cell types reflected in the ratio of the amount

of mesophyll to bundle sheath tissue? (e.g., Expressed as numerical cell ratio, area or volume ratio; related to organelle concentration.) (Chapter 7).

(7) What is the developmental basis and control of the cellular compartmentation of C_4 plant leaves? There is a need, at least, for comparative C_3/C_4 leaf development studies.

(8) Are conflicting species designations in the literature, with respect to photosynthetic pathway, of any significance? viz. *Panicum milioides* (C_4: Downton 1971c; C_3: Moss *et al.* 1969, Wynn *et al.* 1973); *Uniola paniculata* (C_4: Brown and Gracen 1972, Gracen *et al.* 1972; C_3: Smith and Brown 1973. But see Brown and Smith 1974); *Suaeda fruticosa* (C_4: Bender 1971; C_3: Smith and Epstein 1971. See also Welkie and Caldwell 1970). In what way can a plant be a C_3/C_4 intermediate (cf. *Mollugo cerviana*: Kennedy and Laetsch 1974), and how could intermediates be recognised? Genera containing both C_3 and C_4 species could provide clues (see list in Björkman 1973). (Chapter 5.4.2.D, Appendix A.3).

(9) What is the significance of Ellis's (1974a and 1974b) reports that both C_3 and C_4 individuals occur in *Alloteropsis semialata* in South Africa? Do C_3 and C_4 individuals of *A. semialata* occur in disjunct populations, can or do they interbreed, or are there two species? If the claim is substantiated, how is this polymorphism controlled genetically? (By a supergene?)

(10) Have C_3 *Panicum* species evolved from C_4 *Panicum* species? (W. Hartley and G.L. Stebbins - pers. commun.). Is the predominance of a unique type of grass leaf anatomy in many C_3 *Panicum* spp., and in many other C_3 eu-panicoid genera, of any relevance in this context (viz. the "*Isachne*-type" leaf anatomy: Metcalfe 1960)? C_3 *Panicum* spp. are predominantly diploid (cf. tetraploids and higher ploidy levels in other *Panicum* spp. - Gould 1968). Is this relevant?

(11) Is there any meaningful relationship in grasses, between C_4 type, classification, geographical distribution, and climate? For example, the Eragrosteae are a tribe predominantly occurring in arid, hot areas with a high winter temperature, and probably comprise NAD-malic enzyme or PEP carboxykinase species. Species in the Paniceae tend to occur in areas with long wet growing seasons, and are probably mostly NADP-malic enzyme type. (See Hartley 1950, 1958a, 1958b; 1973; Hartley and Slater 1960).

1.2 TERMINOLOGY

Before outlining the actual scope of this thesis in the next section (Chapter 1.3), there is a need to explain some of the terminology used there and in subsequent Chapters.

Initial descriptions of C_4 leaf anatomy were based on plant leaves exhibiting a "Kranz" structure, as first characterised by Haberlandt in 1882 (see Haberlandt 1884; Brown 1975). The term "Kranz leaf anatomy" has tended to become synonymous with "C_4 leaf anatomy", referring to the arrangement of leaf cells in transection where: the "vascular bundles are surrounded by two concentric chlorophyllous layers, an inner parenchyma bundle sheath layer and an outer mesophyll layer. The bundle sheath layer contains specialized plastids" (Downton 1971d). I refer to this type of anatomy (omitting the reference to "specialized plastids") as "classical" C_4 leaf anatomy in the next section (1.3) and in Chapters 4 to 8. Any type of C_4 anatomy deviating from this simple arrangement I term "non-classical".

In plant leaves with "classical" C_4 leaf anatomy, the "mesophyll" and "parenchyma(tous) bundle sheath" (PBS or PS) constitute the two tissue types between which the biochemical events of C_4 photosynthetic metabolism are compartmentalised (e.g., see Downton 1971d). "Mesophyll" and "parenchyma bundle sheath", however, are descriptive histological terms and can be used for both C_4 and C_3 plant leaves. This terminology is used in Chapter 2, where I first question the anatomical characteristics usually associated with C_4 plant leaves. It is worth noting that the term "mesophyll" itself, is recognised by Esau (1965) as referring to all the "ground tissue" in a leaf, including therefore the "parenchyma bundle sheath", whether in a C_3 or a C_4 plant leaf.

The use of the term "chlorenchymatous parenchyma bundle sheath" (CPBS) was introduced by Akita and Miyasaka (1969), and generally, its application enables one to draw attention to the distinction between

C_4 and C_3 plant leaf PBS's. To use the term CPBS or PBS, however, assumes that the chlorenchymatous bundle sheath in C_4 plant leaves is always parenchymatous; an assumption which has yet to be substantiated. For these reasons, I used the term "chlorenchymatous bundle sheath" (CBS) at the time Chapter 3 was written. Biochemists themselves, have always tended to use the simple term "bundle sheath" (BS), but from an anatomical viewpoint this is not specific enough, since it ignores the existence of bundle sheaths other than those already mentioned (e.g., the sheath of cells interpolated between the two chlorenchymatous bundle sheaths in some Cyperaceae).

With the recognition of an increasing number of C_4 species with "non-classical" C_4 leaf anatomy, it became clear that the terms PBS, PS, CPBS, CBS or BS were not always appropriate for referring to the anatomical sites of cells in which the C_4 acid decarboxylase system and Calvin Cycle operate. I therefore introduced (Chapter 4) two terms which are based on function rather than histology: (i) "primary carbon-assimilation" (PCA) cells, and (ii) "photosynthetic carbon-reduction" (PCR) cells, being equivalent to "mesophyll" and "bundle sheath" respectively. These terms are used in Chapter 1.3. and Chapters 4-8.

The PCA/PCR terminology has the advantage of being applicable to all C_4 anatomical types and of being brief for reference to chlorenchymatous cells in plants with "non-classical" C_4 leaf anatomy. Furthermore, for the first time, the "mesophyll" of C_4 plant leaves is referred to by a different label than the "mesophyll" of C_3 plant leaves, and this is appropriate considering their differing functions. Confident designation of cells as PCA or PCR depended upon the development of a technique to recognise cell types functionally; PCR cells can in fact be recognised by immunofluorescent labelling of ribulose-1,5-bisphosphate carboxylase (Chapter 5). Since PCA/PCR designation in leaves of any one species depends upon such evidence, it seemed inappropriate to substitute

the terms PCA and PCR for the anatomical terms employed in Chapters 2 and 3. In the literature on C_4 photosynthetic biochemistry and physiology, the terms (i) mesophyll, and (ii) bundle sheath, PBS, PS, CBS, or CPBS are generally equivalent to PCA and PCR respectively. Brown's (1975) "Kranz" cells are equivalent to PCR cells. The German word "Kranz" means "wreath" or "garland", and in a literal (but not historical) sense, seems no more appropriate than "bundle sheath".

Taxonomic nomenclature of suprageneric taxa could also be a source of confusion for the Gramineae. Grass group names (e.g., "eupanicoid", "festucoid") are used throughout this thesis *sensu* Watson and Clifford (1976).

1.3 SCOPE OF THESIS

The main aim of my work has been to define C_4 plant leaf anatomy, and to relate this to C_4 photosynthetic function. Although it is generally accepted that plants exhibiting C_4 photosynthesis have C_4 leaf anatomy (or "always have the Kranz complex": Hatch and Osmond 1976), the latter has never been adequately defined. My initial survey of grass leaf blade transections, and reports by Bisalputra *et al.* (1969), Black and Mollenhauer (1971), Jacobs (1971), Laetsch (1971), Carolin *et al.* (1973), Crookston and Moss (1973), and Johnson and Brown (1973), had shown that "classical" C_4 leaf anatomy is not manifested in all C_4 species. Subsequent reports (Björkman *et al.* 1974; Chen *et al.* 1974; Ellis 1974a, 1974b; McWilliam and Mison 1974; Oleson 1974; Brown 1975; Carolin *et al.* 1975; Shomer-Ilan *et al.* 1975; Rathnam *et al.* 1976; Chapters 2,4, and 5), and re-examination of "pre-C_4 photosynthesis" reports (Rikli 1895; Holm 1899a, 1899b, 1901; Henrard 1929; Vickery 1935; Burbidge 1946, 1953; Tateoka 1956a, 1956b, 1958, 1961, 1963; Lommasson 1957; Brown 1958; Metcalfe 1960; De Winter 1965) greatly extend the list of species with "non-classical" C_4 leaf anatomy, and extend the types of C_4 leaf anatomy recognised.

Recent articles have called for a redefinition of C_4 plant leaf anatomy (Shomer-Ilan *et al.* 1975; Rathnam *et al.* 1976). Such demands reflect disquiet over the existence of C_4 anatomical variants, and people are beginning to doubt whether there *are* any specific anatomical features associated with C_4 photosynthesis (Jolivet 1976; Kelly *et al.* 1976). Furthermore, the similarity of the leaf anatomy of the putative C_3/C_4 intermediate, *Panicum milioides* (Kanai and Kashiwagi 1975; Chapters 4.3.2, 5.4.2.D) emphasises the need for identification of consistent C_4 anatomical features which are correlated with physiology. Only when this is done, will it be possible to relate anatomy and C_4 photosynthesis in a functional sense.

I have tried in grass leaf blades, to identify features of leaf

anatomy constant to C_4 plants (Chapter 2). Two such features were found (the "maximum lateral cell count" of 2 to 4, and "maximum cells distant count" of 1); and their existence suggested that the functional importance of C_4 leaf architecture may be related, in part, to the necessity for close proximity of PCA and PCR tissues and/or for an "anatomical stoichiometry" compatible with the biochemical stoichiometry of the cellularly compartmented events in C_4 photosynthesis. With respect to the predictive value of the two parameters, for grasses, this has been extensively verified since Chapter 2 was published in Phytomorphology, and the supplementary data are presented in the addendum (Chapter 2.5). Observations on leaf *sheaths* of over 50 grass species (Chapter 6) are consistent with those of the blades with respect to the "maximum lateral cell count" and the "maximum cells distant count". The overall architecture of the two parts of the leaf, however, differs considerably: sheaths of grasses with C_4 blades are apparently C_4, but they usually lack "classical" C_4 anatomy.

While making observations on leaf sheaths, my attention was drawn to sclerenchyma-bundle sheath relations, and to apparent differences between chloridoid (eragrostoid) and eu-panicoid grasses. This led to the recognition of the XyMS character which seems to provide a reliable method of distinguishing C_4 species types (Chapter 3; an addendum, Chapter 3.6, contains XyMS information on 11 species examined subsequent to publication of the original data). C_4 species types also appear to have somewhat different ranges of PCA/PCR volume ratios (Chapter 7). Data on PCA/PCR ratios, however, have as yet been obtained for too few species, and the observations are insufficiently detailed to provide a firm basis for functional-anatomical theories.

Initial results of immunofluorescent labelling of ribulose-1, 5-bisphosphate carboxylase (RuP_2Case), which identifies PCR cells, are introduced in the context of intercellular metabolite transport in C_4 plant leaves, with especial emphasis on species exhibiting "non-classical"

C_4 leaf anatomy (Chapter 4). The XyMS character is also considered in this Chapter, and is probably of significance with respect to the transport of photosynthate from PCR tissue to phloem, and/or to water transport. Chapter 4 is mainly speculative, and the data on which it rests are presented in Chapters 2, 3, and 5.

In situ immunofluorescent labelling of RuP_2Case in leaf blades of 42 species (Chapter 5) confirms earlier conclusions on the location of this enzyme in leaves of species with "classical" C_4 leaf anatomy, and in C_3 and CAM plant leaves. The technique is shown to be especially valuable in identifying PCR cells in leaves of species with "non-classical" C_4 leaf blade anatomy (Chapter 5), and in leaf sheaths (Chapter 6). The labelling results for *Atriplex* hybrids and for the putative C_3/C_4 intermediate, *Panicum milioides*, are of especial interest. Chapter 5 also contains a brief synthesis of my present conception of the essentials of C_4 leaf anatomy. This conception, supported by the observations on leaf sheaths (Chapter 6) and PCA/PCR ratios (Chapter 7), is considered more fully in Chapter 8 in relation to the functional significance of C_4 leaf anatomy.

Since this thesis provides information on the gross architecture of C_4 leaf anatomy, and not ultrastructure, the latter is not discussed (but see review by Laetsch 1974; and subsequent papers: Gutierrez *et al.* 1974a; Oleson 1974; Chapman *et al.* 1975; Hatch *et al.* 1975; Shomer-Ilan *et al.* 1975; Carolin *et al.* 1975).

Appendix 1 outlines very briefly areas for future work which do not appear in the thesis text. In particular, further applications of immunofluorescent labelling are suggested.

1.4 PRESENTATION

Since much of the work described in this thesis has already been published (Chapters 2, 3, and 4) or submitted for publication (Chapter 5), the presentation is basically that of a collection of papers. The format adopted throughout the thesis has been standardised, and conforms to that of the Australian C.S.I.R.O. journals.

The Chapters are all inter-related pieces of work, as outlined in the last section (Chapter 1.3.). The order in which the Chapters are presented, is that in which they were written, since this retains the meaning of much of the cross-referencing in the text, and shows the development of ideas during the course of my study. However, extra cross-referencing has been introduced, especially with respect to illustrations, so that further continuity is provided. For convenience, (i) Chapter headings are referred to by a numbering system, (ii) the cited literature has been pooled, and (iii) the Figures have been pooled and are numbered consecutively throughout the thesis.

Chapters 2 and 3 include addenda which contain data acquired since publication, and which also draw attention to subsequent relevant literature.

The immunofluorescent labelling work described in Chapter 5 has been illustrated, of necessity, with colour photographs. Financial limitations have prevented presentation of better quality ('cibachrome') colour prints or of adequate reproductions of original Ektachrome colour transparencies. Consequently, the colour balance of the subject in the illustrations actually presented does not always conform to that of the original, and this has been unavoidable.

CHAPTER 2

ANATOMICAL PARAMETERS FOR PREDICTING PHOTOSYNTHETIC PATHWAYS OF GRASS LEAVES : THE "MAXIMUM LATERAL CELL COUNT" AND THE "MAXIMUM CELLS DISTANT COUNT"

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2.1 ABSTRACT

Leaf transections from 121 grass species (80 genera) have been examined histologically in relation to features associated with type of photosynthetic pathway. Those most highly correlated with C_4 photosynthesis are the "maximum lateral cell count" (2-4 chlorenchymatous mesophyll cells intervening between PBS cells of laterally adjacent vascular bundles in C_4 species; more than 4 in C_3 species) and the "maximum cells distant count" (in C_4 species no chlorenchymatous mesophyll cell is separated from the nearest PBS cell by more than one other chlorenchymatous mesophyll cell; i.e., the "one cell distant criterion"). These parameter values, especially the latter, provide clues to what may be the true functional significance of the various types of organisation of chlorenchyma found in leaves of C_4 grasses. Using them, photosynthetic pathways are predicted for a further 87 grass species (79 genera) yet to be investigated experimentally.

2.2 INTRODUCTION

The C_4 pathway of photosynthesis is now well documented biochemically and physiologically (Hatch and Slack 1970a, 1970b; Hatch *et al.* 1971; Black 1973; Björkman 1973). C_4 and C_3 species, especially grasses, have also been compared quite widely both histologically and ultrastructurally (see review by Laetsch 1974) and the former are generally held to possess a specialised type of leaf anatomy, referred to as "Kranz anatomy" by Johnson and Brown (1973). This is an interesting phenomenon, which has offered scope for speculation and ideas for experimentation in the realm of functional plant anatomy. The discovery that certain anatomical features generally occur in association with a particular kind of physiology, and may be an integral part of it, emphasizes that details of comparative plant anatomy are relevant to physiology and biochemistry, and that plant anatomists should always work with this in mind.

Various histological and ultrastructural features have been held to characterise C_4 plants and to distinguish them from C_3 plants; i.e., relatively low interveinal distances and thin leaf blades, a low numerical ratio of chlorenchymatous mesophyll cells to parenchymatous bundle-sheath (PBS) cells, radiate chlorenchyma, well developed and thick-walled PBS cells which accumulate abundant starch, characteristic patterns of organelle distribution between PBS and mesophyll cells, and presence of a chloroplast peripheral reticulum in both these cell types (e.g. Chonan 1970, 1972; Takeda and Fukuyama 1971; Monteny 1973; Crookston and Moss 1974; Laetsch 1971, 1974; Huang and Beevers 1972; Imai *et al.* 1973; Johnson and Brown 1973; Black and Mollenhauer 1971). However, all these features are either imperfectly correlated with the photosynthetic pathway as detected physiologically, or are inadequately quantified, or they have been surveyed over too small a sample of species for the reliability of the correlation to be assessed. Moreover, ultrastructural features, while

clearly of great interest from the standpoint of fundamental physiological anatomy, are relatively inaccessible, and do not lend themselves to large-scale surveys (often of necessity involving use of herbarium material) aimed at pinpointing species promising to repay the attentions of experimentalists.

No attribute which fails to correlate absolutely with possession of the C_4 pathway can be structurally essential to it. An imperfect correlation such as that involving chlorenchyma radiateness may arise coincidentally, because like the C_4 pathway itself it is extensively taxonomically correlated (e.g., Smith and Brown 1973); or it may be a slightly imperfect reflection of another, physiologically more critical, feature. In any case, in view of the biological and possible economic significance of the C_4 pathway there is abundant incentive for making further comparative anatomical observations on leaves, in the hope of usefully re-defining aspects of Kranz anatomy or of finding new, physiologically-informative and perhaps more definitive characteristics of it.

While examining preparations of grass leaves made for another purpose (Watson and Milne 1972), it seemed to me that a simple count of interveinal mesophyll cells afforded a relatively unambiguous and theoretically interesting criterion for accurately distinguishing C_3 from C_4 species; and I commenced gathering extensive data. Meanwhile, Crookston and Moss (1974) published a significant short paper, upholding the general validity of distinguishing between C_3 and C_4 grasses in terms of interveinal distances and showing that, for the 20 species of their sample at least, this difference reflects a difference in numbers of mesophyll cells between adjacent vascular bundle sheaths (9-15 in their C_3 species; only 2 in their C_4 species). They also discussed the possible physiological importance of these features. However, their sample was small, and did not include examples for which other Kranz anatomical indicators of photosynthetic pathway break down (e.g., C_3 eu-panicoids with conspicuously

radiate chlorenchyma). Nor did it include species with interveinal "distinctive cells" (Tateoka 1956b) or "circular cells" (Metcalfe 1960) (e.g., *Arundinella*, Fig. 8), which might be expected to provide interpretive difficulties and a test for physiological theories.

2.3 MATERIALS AND METHODS

10-15μm transverse sections were cut by hand from the mid-region of leaf blades from 208 grass species (159 genera), using either fresh or herbarium material. Permanent preparations were made after staining in phenolic Bismarck Brown (Metcalfe 1960).

All preparations were scored wherever possible for the conventional Kranz anatomical characters, namely radiateness of the mesophyll; presence or absence of well-defined parenchymatous bundle sheaths; PBS cells larger than the other mesophyll cells; and (where it was feasible to do so) relative abundance of chloroplasts in chlorenchymatous mesophyll and PBS cells. In addition, they were scored for *numbers of chlorenchymatous mesophyll cells between the PBS cells of laterally adjacent vascular bundles*, with special reference to the maximum number observable in a given leaf (referred to hereafter as the *"maximum lateral cell count"*). The count was made along a straight line from the centre of one bundle to the centre of the next (see Figs. 1,2). Ground tissue other than typical chlorenchymatous mesophyll cells was ignored in the count; e.g. "colourless cells" (found especially in many chloridoid grasses; see Metcalfe 1960), aerenchyma. The *maximum number of cells separating any individual chlorenchymatous mesophyll cell from its nearest PBS cell* was also recorded for each transection, giving rise to the *"maximum cells distant count"* and the *"one cell distant criterion"*.

To test the closeness of association of these anatomical parameters with alternative photosynthetic systems, the anatomical observations have been set against photosynthetic pathway as determined by non-anatomical

means (mainly CO_2 compensation points and carbon isotope ratios; for references, see Table 1).

2.4 OBSERVATIONS AND DISCUSSION

A more or less radiate arrangement of mesophyll cells characterises the vast majority of C_4 grass leaves (e.g., Figs. 1A, 21A). However, *Arundinella nepalensis* (Fig. 8), *Garnotia stricta* (Fig. 1B) and *Triodia pungens* (Figs. 14A, 14B) have non-radiate mesophyll but are C_4, or are presumed to be C_4 from data on other species of the same genus (see Table 1). Furthermore, many grass leaves fall into the category of "indistinctly radiate". Of these, some are C_4 (e.g., *Paspalum dilatatum*; *Zea mays*; *Stenotaphrum secundatum*, Fig. 1C) while others are C_3 or presumed C_3 (e.g., *Arundo donax*; *Catapodium rigidum*; *Danthonia pallida*; Figs. 2A, 2B). C_3 species having clearly radiate mesophyll include many of the C_3 eu-panicoids (e.g., *Coelachne pulchella*; *Cyrtococcum* sp.; *Entolasia stricta*, Fig. 2C; *Isachne globosa*; *Sacciolepis indica*; *Thyridolepis mitchelliana*, Fig. 3A) plus *Cortaderia selloana*.

The PBS is well-defined not only in C_4 species examined but in most C_3 species as well, exceptions being *Holcus lanatus* and *Amphibromus neesii* (Fig. 3C). Furthermore, relatively large PBS cells are by no means diagnostic of the C_4 pathway. Leaves of C_3 species such as *Arundo donax* (Fig. 2A), *Cortaderia selloana*, *Hymenachne amplexicaulis* and *Phragmites australis* (Fig. 2D) have PBS cells larger in transection than the other mesophyll cells, while the PBS cells are smaller than those of the surrounding mesophyll in the C_4 species *Aristida vagans* (outer PBS), *Setaria geniculata* (Fig. 1D) and *Stenotaphrum secundatum* (Fig. 1C). In other species the PBS and surrounding mesophyll cells are much the same size (e.g., *Apluda mutica*, *Hackelochloa granularis*, *Ophiuros exaltatus*, *Paspalidium jubiflorum*). An abundance of chloroplasts in PBS cells is apparent not

only in most C_4 species (see Figs. 1A, 1D), but in such C_3 species as *Danthonia carphoides*, *Hordeum vulgare* (Fig. 3B), *Panicum clandestinum* and *Phragmites australis* (Fig. 2D).

Both the "maximum lateral cell count" and the "maximum cells distant count" are easily recorded even in quite distorted herbarium material, and they provide clearer indications of photosynthetic pathway than do any of the aforementioned criteria. In all C_4 species and presumed C_4 species (Tables 1), the lateral cell count is extremely regular for all bundle-pairs of a section, and its maximum value for a leaf exceeds 4 only in *Arundinella nepalensis* and *Garnotia stricta* (but see below). The regularity of the count draws attention to a little-emphasized aspect of C_4 anatomy, namely that the histological organization in many species is very uniform across the whole blade, even through thickened midribs (e.g., *Echinochloa crus-galli*, *Lepturus repens*, *Saccharum officinarum*). Known and presumed C_3 species, by contrast, exhibit counts which tend to be variable in any one leaf blade; in particular, they are often lower near the leaf margins. Lateral cell counts in C_3 leaves fall in the range 0 to 12, *minimal* counts of 0 having been recorded for *Arundo donax;* of 1 for *Danthonia nivicola*, *D. pallida*, *D. procera* and *Phragmites australis;* of 2 for *Amphibromus neesii*, *Dactylis glomerata*, *Avena fatua*, *Dactylis glomerata*, *Danthonia carphoides*, *Oryza sativa* and *Stipa verticillata;* of 3 for *Triticum aestivum*, *Hymenachne amplexicaulis* and *Stipa teretifolia;* and of 4 for *Poa annua*, *P. helmsii*, *Festuca littoralis*, *Stipa filifolia*, *S. ramosissima* and *S. juncoides*. However, these and all other known or presumed C_3 species (Table 1) exhibit at least some bundle-pairs (usually many) separated by more than 4 (usually many more) chlorenchymatous mesophyll cells (Fig. 2B). In fact, for the vast majority of C_3 leaves, the *minimal* lateral cell count is greater than 6, so that the distinction between C_3 and C_4 grass leaves is usually very clear indeed; cf. Figs. 1D, 2B.

Of the species in Table 1, only 2, viz. *Arundinella nepalensis* (presumed C_4) and *Garnotia stricta* (C_4; Fig. 1B) would be incorrectly assigned by the anatomical criterion of "maximum lateral cell count" as defined above. Species of these genera possess longitudinal strands of cells between the parallel veins of the leaves, variously called "distinctive cells" (Tateoka 1956b; Johnson and Brown 1973), "circular cells" (Metcalfe 1960), and "specialised cells" (Crookston and Moss 1973). They are held to be equivalent to PBS cells, both ultrastructually (Carolin *et al.* 1973) and functionally (Crookston and Moss 1973). Interestingly (see below), if the lateral cell count is made between these cells (Fig. 8) and the PBS of an adjacent vascular bundle, or between laterally adjacent strands, values typical of C_4 species are obtained. Species of *Triodia* (and also *Plectrachne*) exhibit a peculiar leaf anatomy (Burbidge 1946, 1953; Jacobs 1971), which at first sight does not lend itself to lateral cell counting. However, if the count is made between laterally adjacent PBS extensions, across the grooves of the lamina and ignoring epidermal cells, a maximum count of 2 is obtained for *Triodia pungens*. Such a count, if taken to indicate C_4, is compatible with the known physiology of another species of the same genus (*T. irritans*; McWilliam and Mison 1974).

The "maximum cells distant count" seems even more effective than the "maximum lateral cell count" for correctly assigning species as C_3 or C_4, being more readily applicable to leaves of the *Arundinella* and *Triodia* types. Among the known and presumed C_4 species listed in Table 1, I have never found any typically chlorenchymatous mesophyll cell to be separated by more than one cell from the nearest PBS cell (Figs. 1A-E, 10, 11, 12). By contrast, in leaves of known and presumed C_3 species (Table 1), there are always some, and usually many, chlorenchymatous mesophyll cells that are two or more cells distant (Figs. 2A-D, 3A, 3B, 9C). A few C_4 species

(e.g., *Cynodon dactylon*, and especially *Pennisetum villosum* and *Stenotaphrum secundatum*, all with lateral cell counts of 2-4; Figs. 1A,C,E) call for a small qualification. Here, some mesophyll cells are more than one cell distant (Figs. 1A, 1E), but they are generally larger and contain distinctly fewer chloroplasts than do typical mesophyll cells. They are never adjacent to PBS cells, and in *Stenotaphrum secundatum* there is a gradation of these cell types in some parts of the leaf, away from the vascular bundles, into colourless cells.

In Table 2 I list 87 grass species (79 genera) which to my knowledge have not yet been investigated either experimentally or (with 19 exceptions : Carolin *et al.* 1973; Smith and Brown 1973) anatomically with respect to photosynthetic pathway, and nominate them tentatively C_3 or C_4. The physiologically-uninvestigated species of *Panicum* in my sample are included here, rather than in Table 1, since this genus is known to exhibit both C_3 and C_4 species. The assignments in Table 2 are based primarily on "maximum lateral cell counts" and on "maximum cells distant counts", which taken together seem to constitute by far the most accessible and reliable of the known anatomical indicators of photosynthetic pathways in grasses. However, the better-known features of Kranz anatomy when taken in combination are largely in accord.

Amphibromus neesii (Fig. 3C) has leaves with conspicuous adaxial ribbing, and sometimes a lateral cell count of only 4. However, in the domes of the ribs there are usually 3-5 mesophyll cells separating the epidermis from the PBS, so that many are two or more cells distant from the nearest PBS cell. This contrasts with adaxially-ribbed C_4 grass leaves, which never have more than 2 mesophyll cells dorsal to the PBS and where the one cell distant criterion holds. I would therefore expect† this species to be C_3, like all experimentally-tested festucoid grasses. *Pyrrhanthera exigua* provides a similar instance, having a "maximum lateral cell count" of 4. However, its PBS/vascular bundle complexes are small

† confirmed in Chapter 5

relative to the depth of the lamina, so that here too, many mesophyll cells violate the one cell distant criterion. I assign this species too as C_3.

Tables 1 and 2 provide data of considerable taxonomic interest. For example, *Amphipogon* is sometimes placed in the Aristideae with *Aristida* (Prat 1960; Watson and Clifford 1976; cf. De Winter 1965). *Aristida* (C_4; Smith and Brown 1973) is unusual in that it has a double PBS (Fig. 13). I determine *Amphipogon strictus* to be C_3, however, and it does not possess a double PBS. A comparison of Table 1 with the data of Smith and Brown (1973) supports the view that most grass genera are probably consistently either C_3 or C_4, *Panicum* remaining the only known exception. In the absence of a thorough taxonomic revision of generic limits among eu-panicoid grasses, there is no good reason to suppose that *Panicum* as currently entertained is meaningful in any evolutionary sense. The modern *Panicum s.str.* consists of species that have been left over after the removal from *Panicum s.l.* of *Entolasia*, *Echinochloa*, *Ottochloa*, *Paspalum*, etc., and further seemingly inevitable breakup may remove the physiological inconsistency (e.g., see Gould 1974). Therefore, evolutionary speculation about the C_4 pathway should probably be directed towards the eu-panicoid assemblage as a whole, since this is readily defensible as a sound taxonomic grouping. Tables 1 and 2 are in accord with the impression that festucoid grasses are probably all C_3, that all andropogonoids and all definite chloridoids (eragrostoids) are probably C_4; but that the eu-panicoid assemblage takes in substantial suites of both C_3 and C_4 genera (asterisked in Tables). The situation *vis-à-vis* arundinoid and danthonioid grasses remains unclear, because of taxonomic uncertainties clouding this area of grass classification.

The examples provided by unusual leaf forms in *Pyrrhanthera*, *Amphibromus*, *Triodia*, *Plectrachne*, *Arundinella* and *Garnotia* suggest that the "one cell distant criterion" is probably the best histological

predictor of photosynthetic pathway in grasses. Furthermore it may hold fundamental physiological significance. The proximity of the clorenchyma as a whole to the vascular tissue in C_4 species, as reflected by short interveinal distances, thinness of laminae and low lateral cell counts, is thought to facilitate rapid transport of photosynthate from the leaf and to contribute to the alleged greater photosynthetic efficiency of C_4 species (Downton and Tregunna 1968; Monteny 1973; Crookston and Moss 1974). However, the situation in *Plectrachne*, *Triodia*, *Pennisetum* and *Stenotaphrum* and more especially in *Arundinella* and *Garnotia*, *vis-à-vis* the "one cell distant criterion", suggests rather that the true meaning of C_4 anatomical organisation may lie at least in part in the distance (or number of membranes) separating chlorenchymatous mesophyll cells from PBS cells (see also Carolin *et al.* 1973). This interpretation makes sense in terms of the cellular compartmentation of biochemical events in C_4 photosynthesis (see e.g., Hatch and Slack 1970a, 1970b; Black 1973; Kanai and Edwards 1973a, 1973b, 1973c; Gutierrez *et al.* 1974c). The balanced system presumably calls for spatial and biochemical co-ordination, requirements demanding close proximity of the two cell types and also determining the relative abundances of cells and organelles in the mesophyll and PBS. These limitations have not always led to an anatomical arrangement recognisable as Kranz anatomy, but the "maximum cells distant count" of one appears to be a constant characteristic of C_4 grass leaves. It remains to be seen however, and will be interesting to find out, whether the same is true for other plant families.

Table 1. Grasses for which non-anatomical evidence of photosynthetic pathway is available, recorded for "maximum lateral cell count" and "maximum cells distant count"†

"Maximum lateral cell count" 2-4; "maximum cells distant count" 1. C_4.

Andropogon virginicus; *Aristida ramosa*; *A. vagans*; *Arundinella nepalensis*; *Axonopus affinis; *Bothriochloa macra*; **Brachiaria foliosa*; *Cenchrus echinatus; *Chloris truncata*; *Chrysopogon fallax*; *Cymbopogon refractus*; Cynodon dactylon; **Digitaria brownii*; D. sanguinalis; *Distichlis distichophylla*; *Echinochloa crus-galli; Eleusine indica; *Eragrostis benthamii*; *Eremochloa bimaculata*; *Erianthus fastigiatus*; **Eriochloa sp*; Garnotia stricta; Imperata cylindrica; *Leptochloa digitata*; Lepturus repens; Miscanthus sinensis; *Muehlenbergia arisanensis;* *Panicum antidotale; P. laevifolium; *Paspalum dilatatum; P. distichum; *Pennisetum alopecuroides; *P. villosum*; **Rhynchelytrum repens*; Saccharum officinarum; **Setaria geniculata*; Sorghum bicolor; *S. leiocladum*; *Spartina maritima*; *Sporobolus elongatus*; *Stenotaphrum secundatum; Themeda australis; *Thuarea involuta; Tragus australianus; *Triodia pungens*; **Xerochloa barbata*; *X. imberbis*; *X. laniflora*; Zea mays; *Zoysia macrantha*.

/continued

Table 1. cont'd.

"Maximum lateral cell count" >4; "maximum cells distant count" 2 or more. C_3.

Agropyron scabrum; Agrostis avenacea; Anthoxanthum odoratum; Arrhenatherum elatius; Arundo donax; Avena fatua; A. sativa; *Briza minor; Bromus unioloides; Centosteca sp; *Coelachne pulchella;* Cortaderia selloana; **Cyrtococcum sp.;* Dactylis glomerata; *Danthonia alpicola; D. bipartita; D. carphoides; D. dimidiata; D. frigida; D. nivicola; D. nudiflora; D. pallida; D. procera; D. robusta; Deschampsia caespitosa;* Ehrharta erecta; Festuca arundinacea; *F. littoralis; Hierochloë rariflora; Hordeum vulgare;* *Hymenachne amplexicaulis; **Isachne globosa; Leersia hexandra;* Lolium perenne; **Oplismenus aemulus; O. undulatifolius;* Oryza sativa; *Oryzopsis miliacea; *Ottochloa gracillima;* *Panicum clandestinum; Parapholis incurva; *Phalaris aquatica;* Phleum pratense; Phragmites australis; *Phyllostachys sp.;* Poa annua; *P. helmsii; P. queenslandica; *Sacciolepis indica;* Schismus barbatus; Secale cereale; *Stipa aristiglumis; S. bigeniculata; S. blackii; S. columbiana;* S. comata; *S. densiflora; S. elegantissima; S. falcata; S. filiculmis; S. filifolia; S. gigantea; S. ichu; S. juncoides;* S. leucotricha; *S. nervosa; S. pubescens; S. ramosissima; S. teretifolia; S. verticillata;* Triticum aestivum.

† Roman type indicates that non-anatomical evidence of photosynthetic pathway exists for the same species; elsewhere the probable pathway has been deduced from data available for other species of the same genus (experimental data from Akita and Miyasaka 1969; Akita and Tanaka 1973; Akita *et al.* 1969; Bender 1971; Bender and Smith 1973; Chen *et al.* 1970; Downes and Hesketh 1968; Downton and Tregunna 1968; Gutierrez *et al.* 1974c; Hatch *et al.* 1967; Hofstra *et al.* 1972; Krenzer and Moss 1969; McWilliam and Mison 1974; Moss *et al.* 1969; Schöch and Kramer 1971; Smith and Brown 1973; Smith and Epstein 1971). For qualifications regarding *Arundinella, Garnotia, Pennisetum, Stenotaphrum* and *Triodia,* see text.

* Eu-panicoid genera.

Table 2. Probable photosynthetic pathways of grasses on which direct evidence is not available, based on "maximum lateral cell count" and the "one cell distant criterion"

Probable C_4 species ("maximum lateral cell count" 2-4; "maximum cells distant count" 1).

*Apluda mutica; Austrochloris dichanthioides; Brachyachne convergens; Capillipedium spicigerum; *Chamaeraphis hordeacea; *Chionachne cyathopoda; Coelorhachis rottboellioides; Dimeria sp.; Dinebra retroflexa; Diplachne parviflora; Enneapogon nigricans; Enteropogon acicularis; Eulalia fulva; Eustachys distichophylla; Hackelochloa granularis; Hemarthria uncinata; Heterachne brownii; Hyparrhenia filipendula; Iseilema vaginiflorum; Microchloa indica; Ophiurus exaltatus; *Panicum australiense; P. effusum; P. simile; *Paraneurachne muelleri; *Paspalidium gracile; P. jubiflorum; P. rarum; Pheidochloa gracilis; Plectrachne schinzii; Pogonatherum paniceum; Polytoca macrophylla; Polytrias amaura; *Pseudochaetochloa australiensis; Pseudopogonatherum irritans; Rottboellia exaltata; Sclerandrium truncatiglume; Thaumastochloa sp., Tripogon loliiformis; Triraphis mollis.*

/continued

Table 2. cont'd.

Probable C_3 species ("maximum lateral cell count" >4; "maximum cells distant count" 2 or more).

*Amphibromus neesii; Amphipogon strictus; *Ancistrachne sp.; Anisopogon avenaceus; Avellinia michelii; Brachypodium distachyum; Catapodium rigidum; Chionochloa conspicua; Cynosurus echinatus; Desmazeria acutiflora; Deyeuxia quadriseta; Dichelachne sciurea; *Dimorphochloa rigida; Echinopogon cheelii; Elytrophorus spicatus; *Entolasia marginata; E. stricta; E. whiteana; Erythranthera australis; Gastridium ventricosum; Gaudinia fragilis; Holcus lanatus; Lagurus ovatus; Leptaspis banksii; Lophatherum gracile; Lophochloa phleoides; Mibora verna; Microlaena stipoides; Monerma cylindrica; Nassella trichotoma; *Neurachne alopecuroidea; Notochloë microdon; *Panicum milioides; Pentapogon quadrifidus; Pentaschistis airoides; Polypogon monspeliensis; Potamophila parviflora; *Pseudechinolaena polystachya; Psilurus incurvus; Pyrrhanthera exigua; Sclerochloa dura; Sphenopus divaricatus; Tetrarrhena laevis; *Thyridolepis mitchelliana; Thysanolaena maxima; Trisetum spicatum; Vulpia bromoides.*

* Eu-panicoid genera.

2.5 ADDENDUM

Since March 1975, when this Chapter was submitted for publication, I have made observations on a further 74 grass species and 19 genera. These have been assigned as C_4 (64 spp. : Table 3) or C_3 (10 spp.) using the "maximum lateral cell count" and the "maximum cells distant count".

Of the 10 species designated C_3, two have been tested for localisation of ribulose-1,5-bisphosphate carboxylase (RuP_2Case) using immunofluorescent labelling (Chapter 5.4.2.; Fig. 19A), and their leaves exhibit a C_3 labelling response (*Panicum pygmaeum*, *Poa sieberana*). *Micraira subulifolia* exhibits a low $\delta^{13}C$ value (Smith and Brown 1973), typical of C_3 species, and a further four species belong to genera containing other species which have been recognised as C_3 (*Danthonia linkii* var. *fulva*, *Festuca asperula*, *Hordeum leporinum* and *Stipa neesiana*) (Krenzer *et al.* 1975; Troughton *et al.* 1974). The remaining three species assessed to be C_3 are *Aira cupaniana*, *Cleistochloa subjuncea*, and *Panicum trichoides*. In total, I have examined 127 C_3 species in this thesis (86 genera).

Of the 64 species designated C_4 (Table 3), 49 have been classed as C_4 by other workers, and for two more, immunofluorescent localisation of RuP_2Case gives a C_4 labelling response (Chapter 5.4.2 : *Aristida behriana*, *A. biglandulosa*). In no case has there been a conflict between anatomical prediction and conclusions based on experiment. Most, but not all of the C_4 species in Table 3, are also listed in Chapter 3 (Table 1), but are presented here for convenience. In total, I have examined 154 C_4 grass species (92 genera) in leaf blade transection (this Chapter : Tables 1,2,3).

Some species and genera listed in Table 2 (this Chapter), where I predicted photosynthetic pathway for 87 species, have been investigated experimentally since March 1975, and the predictions were verified in all cases.

(i) C_4 : *Iseilema vaginiflorum*, *Panicum effusum*, and species of *Apluda*, *Enteropogon*, and *Hyparrhenia* other than those in Table 2.

(ii) C_3 : *Amphibromus neesii*, *Entolasia stricta*, *Holcus lanatus*, *Microlaena stipoides*, *Polypogon monspeliensis*, *Thyridolepis mitchelliana*, and species of *Brachypodium* and *Trisetum* other than those in Table 2.

(Krenzer *et al.* 1975; Raghavendra and Das 1976; Troughton *et al.* 1974; Chapter 5.4.2.).

Predictions for 14 species in Table 1 (this Chapter), which were based on results for other species of the same genus, have also since been verified:

(i) C_4 : *Aristida ramosa*, *Arundinella nepalensis*, *Brachiaria foliosa*, *Chloris truncata*, *Digitaria brownii*, *Pennisetum villosum*, *Rhynchelytrum repens*, *Sporobolus elongatus*.

(ii) C_3 : *Briza minor*, *Danthonia bipartita*, *Deschampsia caespitosa*, *Isachne globosa*, *Oryzopsis miliacea*, *Stipa verticillata*.

(Krenzer *et al.* 1975; Troughton *et al.* 1974; Chapter 5.4.2.).

Table 3. Grasses assigned as C_4 on the basis of the "maximum lateral cell count" and "maximum cells distant count" (observed since Chapter 2 was published). All species have independently been recognised as C_4 on non-anatomical evidence (Downton 1975; Gutierrez *et al.* 1974a, 1976; Krenzer *et al.* 1975; Sankhla *et al.* 1975; Troughton *et al.* 1974; Chapter 5.4.2.), *except* those asterisked.

**Acrachne verticillata, Alloteropsis semialata*†*, Andropogon scoparius, *Anthephora acuminata, Aristida behriana, A. biglandulosa, Bouteloua gracilis, Buchloë dactyloides, Cenchrus pauciflorus, Chloris cucullata, C. distichophylla, C. gayana, Cynodon arcuatus, Echinochloa colonum, E. frumentacea, Eleusine coracana, *Elionurus citreus, *Eragrostiella bifaria, Eragrostis cilianensis, E. curvula, Eriochloa pseudoacrotricha, *Ischaemum australe, Melinis minutiflora, Muehlenbergia schreberi, Panicum bergii, P. bulbosum, P. capillare, P. coloratum, P. coloratum var. makarikariense, P. decompositum, P. deustum, P. hallii, P. maximum, P. miliaceum, P. molle, *P. muelleri, P. obtusum, P. repens, *P. seminudum, P. stapfianum, P. texanum, P. turgidum, P. virgatum, *Paractenium novae-hollandiae, Paspalum conjugatum, P. notatum, Pennisetum clandestinum, P. purpureum, P. typhoides, *Plagiosetum refractum, Setaria italica, S. verticillata, S. viridis, Sorghum halepense, S. sudanense, Spinifex hirsutus, *Sporobolus africanus, S. airoides, S. cryptandrus, *S. diander, S. fimbriatus, Urochloa mosambicensis, U. panicoides, *Whiteochloa semitonsa, *Zygochloa paradoxa.*

† I examined the C_4 form of this species (cf. Ellis 1974a; Brown 1975).

CHAPTER 3

C_4 GRASSES: AN ANATOMICAL CRITERION FOR DISTINGUISHING BETWEEN NADP-MALIC ENZYME SPECIES AND PCK OR NAD-MALIC ENZYME SPECIES

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3.1 ABSTRACT

Transections of leaf blades from 46 C_4 grass species of known C_4 type were recorded for presence (XyMS+) or absence (XyMS-) of cells intervening between metaxylem vessel elements and laterally adjacent chlorenchymatous bundle sheath cells of primary lateral vascular bundles. A perfect correlation, with only "NADP-ME type" species being XyMS-, indicated that XyMS type serves as a useful predictor of C_4 species type. The likely C_4 type of 257 biochemically untyped grass species was accordingly deduced, by direct observation (94 spp.) or from Metcalfe's (1960) anatomical descriptions (163 spp.). All 124 C_3 species examined, including C_3 eu-panicoids, proved to be XyMS+.

3.2 INTRODUCTION

Three variations of C_4 photosynthesis, which seem to be species-specific, are currently recognised. Species are classed as "NADP-ME type" (= Group I = NADP-malic enzyme species), "NAD-ME type" (= Group III = NAD-malic enzyme species), or "PCK type" (= Group II = PEP-carboxykinase species), depending on the reaction sequence for C_4 acid decarboxylation in the bundle sheath cells of leaf blades (Gutierrez *et al.* 1974a, 1974c; Hatch *et al.* 1975). Recognition of three C_4 types was foreshadowed by discovery of differences in $^{14}CO_2$ labelling patterns between C_4 species (e.g., Hatch and Slack 1966; cf. Chen *et al.* 1971), and Downton (1970, 1971a) classified C_4 species into "malate formers" and "aspartate formers" on this basis. Of the 11 species he designated as "malate formers", seven have subsequently been examined in more detail with respect to C_4 acid decarboxylation, and all have proved to be NADP-ME type; while of 16 "aspartate formers", eight are now recognised as NAD-ME type, and three as PCK type.

Certain physiological characteristics may be correlated with C_4 type: e.g., whether or not there is an oxygen-insensitive post-illumination carbon dioxide burst, and the kind of post-illumination carbon dioxide release transient exhibited (Downton 1970; Brown and Gracen 1972). Correlations with various ultrastructural features of the bundle sheath cells are also becoming increasingly apparent, at least for grasses: e.g., chloroplast position (Downton *et al.* 1970; Black and Mollenhauer 1971; Downton 1971b; Brown and Gracen 1972; Gutierrez *et al.* 1974a, 1974b; Hatch *et al.* 1975), degree of granal stacking in the chloroplasts (e.g., Downton *et al.* 1970; Carolin *et al.* 1973; Gutierrez *et al.* 1974b), and mitochondrial "concentration" and structure (e.g., Black and Mollenhauer 1971; Downton 1971b; Laetsch 1971; Newcomb and Frederick 1971; Carolin *et al.* 1973; Hatch *et al.* 1975).

Much of the work on C_4 types has been done with grasses, and of 61 species so far examined 29 are NADP-ME type, 21 are NAD-ME type, and 11 are PCK type (Edwards *et al.* 1971; Kanai and Black 1972; Huber *et al.* 1973; Gutierrez *et al.* 1974a, 1974c; Hatch and Kagawa 1974; Hatch *et al.* 1975). The distribution of these types within the grass family is of interest both taxonomically and phylogenetically. All known NADP-ME type species are eu-panicoid or andropogonoid, with the exception of *Aristida purpurea*(classed as aristidoid by Smith and Brown 1973). Known NAD-ME type and PCK type species are mostly chloridoid (eragrostoid), but these types also occur in the genera *Panicum* and *Urochloa* (eu-panicoid, tribe Paniceae). *Panicum*, in fact, contains all three C_4 type species, and C_3 species as well (e.g., see Gutierrez *et al.* 1974a). Although there is a general correlation of C_4 type with taxonomy at subfamily level, the existence of exceptions precludes accurate prediction of type from systematics alone.

Gutierrez *et al.* (1974a, Table 1) have compiled evidence which suggests that the three C_4 types can be distinguished ultrastructurally by using a combination of two bundle sheath cell chloroplast characters; viz., the degree of granal stacking, and chloroplast position. However, it would be useful if readily accessible anatomical characters, observable in hand-cut leaf blade sections, could be employed. Brown (1960) has demonstrated that chloroplast position can be reliably ascertained using the light microscope. There is good agreement between his observations and subsequent ultrastructural reports, so that it seems possible that, in fresh material, NAD-ME type species could be distinguished from other types with the light microscope by using this character. The coincidence between Brown's (1960) sample and biochemically "typed" species is small, however, so that further testing is required. The same is true for differences among C_4 grasses in parenchyma bundle sheath cell shape (as observed in longitudinal section) noted by Brown (1974), so that the

significance or diagnostic value of this character in relation to C_4 type remains unknown. To a very large extent, the variations found within C_4 grasses in both bundle sheath cell chloroplast position and cell shape reflect broad cytological differences between the eu-panicoid and andropogonoid grasses on one hand, and chloridoid (eragrostoid) grasses on the other (see Brown 1960, 1974).

Of other anatomical features of leaf blades generally held to distinguish eu-panicoids or andropogonoids from chloridoids (eragrostoids), one seems of particular interest with respect to C_4 type. Previous records concerning the mestome sheath (MS) (e.g., Schwendener 1890; Brown 1958) suggest that the taxonomic distribution pattern of C_4 grasses in which the sheath is present may closely parallel that of NAD-ME and PCK type species. I have therefore examined a large proportion of those species which have been biochemically "typed", to see if the MS character can be used to distinguish NADP-ME type species from NAD-ME and PCK type species. In practice, serious difficulties are encountered if one attempts to record grass leaves as positive or negative for the MS, not the least of which is that MS cells have never been adequately characterised anatomically, developmentally, or histochemically (but see Schwendener 1890; Van Fleet 1950; O'Brien and Carr 1970). If the criteria generally used for recognising the MS are adopted (viz., cells which are small and thick-walled, and which form a sheath between the parenchymatous bundle sheath and vascular tissue), considerable intra- as well as interspecific variation is found, not only in presence and completeness of the MS, but also in the nature of the constituent cells. I have, therefore, selected a specific aspect of the MS which can be easily and unambiguously recorded, and which appears to be of diagnostic value with respect to C_4 type, being common to both NAD-ME type and PCK type species, but absent from NADP-ME type species.

3.3 METHODS

Transverse hand sections 10-15 μm thick were cut from the mid-region of leaf blades from 140 C_4 grass species (83 genera), either fresh or herbarium material being used. This sample included 46 species (out of the 61 known to me) that have been biochemically typed. Leaf blades of 124 C_3 species were also sectioned. Temporary or permanent mounts were made after staining in phenolic Bismarck Brown. Sections of C_4 species were scored for the *presence (XyMS+) or absence (XyMS-) of cells between the metaxylem vessel elements and laterally adjacent chlorenchymatous bundle sheath (CBS or "Kranz") cells in the primary lateral vascular bundles* (i.e., in the largest lateral vascular bundles having both metaxylem and protoxylem). C_4 species were then classed as XyMS+ when the presence of intervening cells was recorded in *all* primary lateral vascular bundles, and as XyMS- when metaxylem vessels were in direct contact with clorenchymatous bundle sheath cells in *at least some* primary lateral vascular bundles. The same approach was adopted for C_3 species, except that scoring was done between the metaxylem vessels and parenchymatous bundle sheath cells (the PBS cells contain few chloroplasts in most C_3 species).

3.4 OBSERVATIONS

In Table 1, I have grouped the C_4 species examined as XyMS+ (e.g., Figs. 4A, 4B, 5A, 6A, 6B) or XyMS- (e.g., Figs. 4C, 4D, 6C, 6D). Of the biochemically "typed" species in my sample, all 27 NAD-ME or PCK type species are, without exception, XyMS+ (asterisked XyMS+ spp.; Table 1), and in these, cells intervene between the metaxylem vessels and CBS cells in *all* primary lateral vascular bundles (e.g., Figs. 4A, 4B, 5A). By contrast, the 19 known NADP-ME type species examined are all XyMS- (double-asterisked XyMS- spp.; Table 1). In 18 of the latter,

all primary lateral vascular bundles were found to be XyMS- (e.g., Figs. 4C, 4D), but in *Saccharum officinarum* (andropogonoid) some vascular bundles were XyMS-, some XyMS+, and in others the metaxylem vessels were in direct contact with CBS cells on one side of the vascular bundle only (Fig. 5B).

The variable XyMS condition as found in *S. officinarum* was encountered in leaf blades from three other species only (Table 1: *Chrysopogon fallax*, *Erianthus fastigiatus*, *Imperata cylindrica*, Fig. 5C), these too being andropogonoid. I group these with XyMS-, rather than with XyMS+ species, since all other andropogonoids (Table 1; Figs. 6C and 6D) are XyMS-, and since all ultrastructural and biochemical evidence points to andropogonoids being NADP-ME type (see refs. above; Edwards *et al.* 1971; Gutierrez *et al.* 1974a). I emphasise that for all other species sampled, the XyMS condition within a leaf blade was consistent for all primary lateral vascular bundles observed.

In XyMS- species, the CBS ("Kranz") cells of primary lateral vascular bundles, but not necessarily of others, are generally equal to, or smaller than, the adjacent mesophyll cells in transectional area (e.g., Figs. 4C, 4D, 6C, 6D, 10). By contrast, CBS cells of the primary lateral vascular bundles of XyMS+ species are generally larger in transectional area than cells of adjacent mesophyll (e.g., Figs. 4B, 6A, 11A, 21A). These observations are probably related to CBS cell shape (see Brown 1974), since they reflect radial and tangential dimensions.

The 124 C_3 species examined were all XyMS+ (e.g., Figs. 3C, 5D, 9C) with the possible exception of *Coelachne pulchella*. These species include 117 C_3 species listed in Chapter 2 (Tables 1 and 2), plus *Aira cupaniana*, *Danthonia linkii* var. *fulva*, *Festuca asperula*, *Hordeum leporinum*, *Panicum pygmaeum*, *P. trichoides*, and *Stipa neesiana*. The sample is widely representative of the C_3 grass subfamilies, with the exception of the

bamboos, and includes species from 12 C_3 eu-panicoid genera as well as from *Panicum* (Fig. 19A). Considering the whole sample of grasses examined, the implication is that C_4 NADP-ME type species alone are XyMS-.

It has proved feasible to interpret the exhaustive and admirable descriptions of leaf transverse sections of both C_3 and C_4 species provided by Metcalfe (1960), in order tentatively to extend my list of XyMS- and XyMS+ species (Table 2). Species dealt with by him I assigned as C_4, using the characters (i), vascular bundles "crowded", reflecting a low lateral cell count (see Chapter 2), and (ii), chlorenchyma "radiate" or "distinctly radiate". These were then assigned as XyMS- if Metcalfe described the bundle sheaths as "single"; and as XyMS+ when he records vascular bundles as having "double" sheaths and/or having the "inner sheath complete", or notes large vascular bundles with double sheaths. Data obtained in this way (Table 2) are likely to incorporate errors, notwithstanding the omission of those species where interpretation of Metcalfe's descriptions presents difficulties. Nevertheless, of the species interpreted as C_4 and listed in Table 2, 24 (in roman type) have been more rigorously examined in the C_4/C_3 context, and are all C_4 (Moss 1968; Downton 1971c; Brown and Gracen 1972; Imai *et al.* 1973; Smith and Brown 1973). Two of them (asterisked, Table 2) have, in fact, been biochemically "typed", and the results are as expected from the XyMS pattern. Furthermore, out of 42 species described by Metcalfe (1960) and also examined by me (spp. in roman type, Table 1), in only two is there a discrepancy between the XyMS condition as observed by me, and that deducible from Metcalfe's accounts. For *Panicum capillare*, my material is XyMS+, in conformity with this species being NAD-ME type (Gutierrez *et al.* 1974a), but Metcalfe (1960) recorded its bundle sheaths as "single". For *Imperata cylindrica*, Metcalfe describes the bundle sheaths as "double", whereas in material I examined, a variable XyMS condition was found. Since I find such a condition in three other andropogonoid species (see above), it

might seem that andropogonoids could have been incorrectly assigned as XyMS+ from Metcalfe's data. In fact, *no* XyMS+ species listed in Table 2 *are* andropogonoid, so this has not happened. Furthermore, four species, not observed by me, and belonging to three of the four genera in which I found the variable XyMS, are described as having single bundle sheaths by Metcalfe (i.e., I class them XyMS-).

3.5 DISCUSSION

The XyMS condition is readily ascertained in hand-cut transverse sections of leaves, even of herbarium material (Figs. 4A, 5A), and data on 46 species from 20 genera (Table 1) show a perfect correlation between C_4 type and XyMS classification. The association holds good even where the sample cuts across well-established subfamilial groupings, so this result does not reflect mere taxonomic sampling bias. The eu-panicoid species *Urochloa panicoides* and 12 *Panicum* species are XyMS+ *and* NAD-ME or PCK type (e.g., Figs. 4A, 4B), in contrast to other biochemically typed eu-panicoids, including two *Panicum* species, which are all XyMS- and NADP-ME type (e.g., Figs. 4C, 4D). Furthermore, the XyMS classification is perfectly correlated with those CBS cell chloroplast features which appear to characterise the C_4 types (c.f. Tables 1 and 2; Downton 1971b; Brown and Gracen 1972; Carolin *et al.* 1973; Johnson and Brown 1973; Gutierrez *et al.* 1974a). With respect to Downton's (1970) work, however I note that *Panicum laevifolium* was reported as a "malate former", while I have found it to be XyMS+.

Turning now to species in Tables 1 and 2 for which only anatomical observations exist, it seems reasonable to anticipate that those designated XyMS- are probably NADP-ME type (e.g., *Eremochloa bimaculata* and *Themeda australis;* Figs. 6C, 6D), the remainder (XyMS+) being NAD-ME or PCK type (e.g., *Eragrostis benthamii* and *Triodia pungens;* Figs. 6A, 6B, 14B). The reliability of the predictions is open to testing, and the possible

existence of variations within the C_4 types, or of gradations from one type to another, cannot be ruled out. Meanwhile, records of presence or absence of the XyMS character raise interesting functional-anatomical and taxonomic questions.

Taxonomic discussion of the distribution of the XyMS in grasses, and of the biochemical variants of C_4 photosynthesis which it appears to characterise, is severely hampered by the absence of a modern, comprehensive classification. Nevertheless, some broad generalisations are possible and a brief taxonomic survey of the XyMS character is worthwhile, because it serves to pinpoint grasses which might repay biochemical and/or taxonomic study.

In so far as the XyMS condition can be relied upon as a predictor, Tables 1 and 2 considerably extend, and to a large extent confirm the taxonomic picture regarding C_4 types as already revealed by biochemical studies: i.e., XyMS+ species (presumed NAD-ME or PCK type) are mostly chloridoid (eragrostoid) (94 out of 136), while most XyMS- species (presumed NADP-ME type) are eu-panicoid, andropogonoid, or belong to the genus *Aristida* (143 out of 167). However, it appears from Metcalfe's (1960) descriptions that a few chloridoids (eragrostoids) are XyMS- (species of *Latipes*, *Leptocarydion*, *Oropetium*, and *Leptochloa uniflora*: Table 2) and it would be interesting to have biochemical information on them. Tables 1 and 2 also show that XyMS+ species (by inference NAD-ME or PCK type) are commoner among the eu-panicoids than available biochemical data suggest (*Brachiaria* - 4 spp., *Melinis minutiflora*, *Panicum* - 20 spp., *Rhynchelytrum repens*, *Thuarea involuta*, *Urochloa* - 2 spp.); and it is noteworthy that C_3 eu-panicoids, like other C_3 species, are XyMS+.

Panicum is currently the only genus biochemically shown to include both NADP-ME (XyMS-) and NAD-ME or PCK type (XyMS+) species (Figs. 4A, 4B, 4C), but four other genera appear to be inconsistent for the XyMS character (*Brachiaria*, *Leptochloa*, *Lepturus*, *Loudetiopsis*), and these need investigating.

As far as the available data go, however, most grass genera are consistently XyMS+ or XyMS-. Anatomical or biochemical inconsistencies within genera, or indeed at other hierarchical levels, may be indicative of a need for taxonomic revision, or of identificatory errors, and any evolutionary speculation should be viewed in this light. In particular, the eu-panicoids, and especially *Panicum* and related genera, are in need of taxonomic revision on a world scale, while the arundinoid grasses are a taxonomically heterogeneous assemblage of genera. Indeed C_4 arundinoid-danthonioid genera listed in Tables 1 and 2 are heterogeneous with respect to the XyMS character (XyMS-: *Arundinella* (Fig. 8), *Danthoniopsis*, *Garnotia* (Fig. 1B), *Loudetiopsis* - 4 spp., *Tristachya;* XyMS+: *Loudetia*, *Loudetiopsis* - 6 spp., *Triraphis*). Anatomical and biochemical information will no doubt contribute to understanding the affinities of these and other taxonomically puzzling genera such as *Aristida* (Fig. 24A) and *Fingerhuthia* (XyMS-), and *Monelytrum*, *Pheidochloa*, *Pommereulla*, and *Triodia* (Fig. 14B) (XyMS+).

Many questions arise concerning the function (e.g., see O'Brien and Carr 1970) and development of cells in the MS position, especially in relation to (i) their presence in the XyMS position in primary lateral vascular bundles of NAD-ME and PCK type, but not of NADP-ME type C_4 species, hinting perhaps at functional differences involving transport of assimilate to phloem-loading areas (see Kuo *et al.* 1974); (ii), the variable XyMS condition in large vascular bundles of some andropogonoid species (Fig. 5B); and (iii), their variable occurrence in vascular bundles of different sizes within one leaf blade. Brown (1975) has suggested that in some C_4 grass species, apparently those which I assign as XyMS-, the chlorenchymatous ("Kranz") bundle sheath is homologous to the mestome sheath i.e., it is not the homologue of the CBS in XyMS+ species. The unique bundle sheath arrangements in some grasses, especially *Alloteropsis semialata* (see Ellis 1974a) and *Aristida* spp. (with two chlorenchymatous sheaths:

Fig. 24A), are particularly interesting in these connections. However, better appreciation of the significance of variations in bundle sheath anatomy in the grass family awaits more developmental, physiological, and biochemical work of a comparative nature.

Table 1. C_4 grass species observed, classified as XyMS+ or XyMS-.[†]

XyMS+ (66 spp; 34 genera):

*Acrachne verticillata; Austrochloris dichanthioides; *Bouteloua gracilis; Brachiaria foliosa; Brachyachne convergens;* *Buchloë dactyloides; *Chloris cucullata; *C. distichophylla: *C. gayana; C. truncata; *Cynodon arcuatus;* *C. dactylon; *Dinebra retroflexa; Diplachne parviflora;* Distichlis distichophylla; Eleusine coracana; *E. indica; *Enneapogon nigricans; Enteropogon acicularis; Eragrostis benthamii;* *E. cilianensis; *E. curvula; *Eriochloa australiensis; Eustachys distichophylla; Leptochloa digitata;* Lepturus repens; Melinis minutiflora; *Microchloa indica; Muehlenbergia arisanensis; *M. schreberi; Panicum australiense; *P. bergi;* *P. capillare; *P. coloratum; *P. coloratum var. makarikariense; *P. decompositum; P. deustum; P. effusum; *P. hallii; P. laevifolium;* *P. maximum; *P. miliaceum; *P. molle; P. muelleri;* P. repens; *P. seminudum; *P. stapfianum; *P. texanum; *P. turgidum;* P. virgatum; *Pheidochloa gracilis;* Rhynchelytrum repens; Spartina maritima; Sporobolus africanus; *S. airoides; *S. cryptandrus;* S. diander; *S. elongatus; *S. fimbriatus; Thuarea involuta; Tragus australianus; Triodia pungens; Tripogon loliiformis; Triraphis mollis; *Urochloa panicoides;* Zoysia macrantha.

/continued

Table 1. (cont'd.)

XyMS- (74 spp.; 50 genera):

*Alloteropsis semialata; **Andropogon scoparius; **A. virginicus;* Apluda mutica; *Aristida biglandulosa; A. ramosa; Arundinella nepalensis; Axonopus affinis; Bothriochloa macra; Capillipedium spicigerum; Cenchrus echinatus;* **C. pauciflorus; *Chamaeraphis hordeacea; Chionachne cyathopoda; Chrysopogon fallax; Coelorachis rottboellioides;* Cymbopogon refractus; *Digitaria brownii; **D. sanguinalis; Dimeria* sp.; **Echinochloa colonum; **E. crus-galli; ***E. frumentacea; Elionurus citreus; Eremochloa bimaculata; Erianthus fastigiatus; Eulalia fulva; Garnotia stricta; Hackelochloa granularis;* Hemarthria uncinata; Hyparrhenia filipendula; Imperata cylindrica; Ischaemum australe; *Iseilema vaginiflora; Miscanthus sinensis; Ophiurus exaltatus; **Panicum antidotale; **P. bulbosum;* P. obtusum; *Paraneurachne muelleri; Paspalidium jubiflorum; P. rarum;* Paspalum conjugatum; *P. dilatatum;* P. distichum; ***P. notatum; Pennisetum alopecuroides;* P. clandestinum; ***P. purpureum; **P. typhoides; P. villosum;* Pogonatherum paniceum; *Polytoca macrophylla; Polytrias amaura; Pseudochaetochloa australiensis; Pseudopogonatherum irritans;* Rottboellia exaltata; **Saccharum officinarum; *Sclerandrium truncatiglume; Setaria geniculata;* **S. italica; ***S. verticillata;* **S. viridis; ***Sorghum bicolor;* S. halepense; S. leiocladum; ***S. sudanense;* Stenotaphrum secundatum; *Thaumastochloa* sp.; Themeda australis; *Xerochloa barbata; X. imberbis; X. laniflora;* **Zea mays.

† Species indicated by an asterisk (27 XyMS+ spp.) have been biochemically determined to be "NAD-ME type" or "PCK type". Species indicated by double asterisks (19 XyMS- spp.) have been biochemically determined to be "NADP-ME type". Species in roman type were also described by Metcalfe (1960).

Table 2. Species described by Metcalfe (1960) and assigned by me as C_4.
Classified as XyMS+ or XyMS-.†

XyMS+ (70 spp; 35 genera):

Astrebla squarrosa; *Bouteloua curtipendula; *Brachiaria distichophylla; B. paspaloides; B. reptans; Calamovilfa longifolia; Chloris barbata; C. filiformis; C. pycnothrix; C. robusta;* Dactyloctenium aegyptium; *D. giganteum; Desmostachya bipinnata; Enneapogon cenchroides; Eragrostiella bifaria; Eragrostis aspera;* E. chloromelas; *E. diplachnoides; E. echinochloidea; E. gangetica; E. unioloides; Eustachys paspaloides;* Gymnopogon ambiguus; *G. delicatulus; G. foliosus; G. spicatus; Indopoa paupercula; Leptochloa coerulescens; Lepturella aristata (=Oropetium aristatum); L. capensis; Lepturus hildebrandtii; L. mildbraedianus; L. xerophilus; Loudetia superba; Loudetiopsis chevalieri; L. chrysothrix; L. glabrata; L. kerstingii; L. ternata; L. villosipes; Microchloa caffra; Monelytrum luederitzianum;* Muehlenbergia racemosa; *Munroa mendocina; M. squarrosa; Pappophorum alopecuroideum; P. pappiferum; Perotis indica; P. patens; P. rara; Pogonarthria squarrosa; P. tuberculata; Pommereulla cornucopiae;* Schedonnardus paniculatus; *Scleropogon brevifolius; Spartina townsendii; Sporobolus indicus; S. molleri;* *S. poiretii; *S. pyramidalis;* S. wrightii; *Tetrapogon villosus; Tragus berteronianus; Tridens flava; T. grandiflorus; T. muticus; Triraphis pumilio; Urochloa pullulans.*

/continued

Table 2. (cont'd.)

XyMS- (93 spp.; 55 genera):

Alloteropsis cimicina; Amphilophis affinis; A. intermedia; Andropogon amplectens; A. schirensis; A. venustus; Anthephora pubescens; *Arthraxon quartinianus; Arundinella metzii; A. villosa; Beckeropsis uniseta; Bothriochloa caucasica; B. pertusa; Brachiaria deflexa; B. jubata; Capillipedium parviflorum; Cenchrus biflorus;* C. ciliaris; Chrysopogon gryllus; *C. zeylanicus; Cleistachne sorghoides;* Coix lachryma-jobi; Cymbopogon citratus; *C. giganteus; C. martini; C. nardus; C. validus; Danthoniopsis humbertii; D. minor; D. stocksii, D.viridis;* Dichanthium aristatum; *D. polyptychum; D. sericeum; Digitaria borbonica; D. brazzae; D. horizontalis; D. milanjiana; Dimeria thwaitesii; Elionurus chevalieri; E. hirtifolius; Eremochloa muricata; Erianthus hostii;* Euchlaena perennis; *Euchlaezea mertonensis; Euclasta condylotricha; Eulalia geniculata; E. phaeothrix; Fingerhuthia africana; Garnotia courtallensis;* Heteropogon contortus; *Hyparrhenia dissoluta; Ischaemum commutatum; I. laxum; I. santapaui; Iseilema membranacea; Latipes senegalensis; Leptocarydion vulpiastrum; Leptochloa uniflora;* Lepturus radicans; *Loudetiopsis ambiens; L. capillipes; L. purpurea; L. tristachyoides; Microstegium ciliatum; Miscanthus condensatus;* M. sacchariflorus; *Oropetium africanum; O. thomaeum; Paspalidium geminatum;* Paspalum commersonii; *P. paniculatum; Pennisetum macrourum; Pollinia fulva; Pseudanthistiria umbellata; Saccharum benghalense; Schizachyrium jeffreysii; S. obliquiberbe;* Setaria barbata; S. glauca; *S. plicata;* Snowdenia polystachya; *Stenotaphrum dimidiatum; Themeda avenacea; T. quadrivalvis; T. tremula;* T. triandra; *Triplopogon spathiflorus;* Tripsacum dactyloides; *Tristachya hispida; T. inamoena; Vetiveria elongata; V. zizanioides.*

† Species in roman type have previously been recognised as C_4 by other authors (see text). The two XyMS+ spp. indicated by an asterisk have been biochemically determined to be "PCK type". For 42 additional species described by Metcalfe and also observed by me see Table 1.

3.6 ADDENDUM

The following 11 species, designated C_3 or C_4 (see Chapter 2.5) were observed after submission of this Chapter for publication in October 1975, and are here classed as XyMS+ or XyMS-.

C_4, XyMS+: *Eriochloa pseudoacrotricha*, *Urochloa mosambicensis*.

XyMS-: *Anthephora acuminata*, *Aristida behriana*, *Plagiosetum refractum*, *Spinifex hirsutus**, *Whiteochloa semitonsa*, *Zygochloa paradoxa* (♀).

C_3, all XyMS+: *Cleistochloa subjuncea*, *Micraira subulifolia*†, *Poa sieberana*.

* Exhibits a variable XyMS condition, like *Saccharum officinarum* (Fig. 5B).

† The leaf in this C_3 species appears to exhibit a double chlorenchymatous bundle sheath (cf. *Aristida*, C_4: Fig. 24A).

There has been little further biochemical typing of C_4 grass species since October 1975, with the significant exception of Gutierrez *et al.*'s (1976) work. They report a further 20 PCK type species: *Brachiaria* (11 spp.), *Eriochloa* (5 spp.), *Panicum* (1 sp.), and *Urochloa* (3 spp.). Of these, I have observed leaf transections of *Eriochloa pseudoacrotricha* and *Urochloa mosambicensis* (above), and both are XyMS+ as expected. A further species, *Urochloa pullulans*, was predicted to be XyMS+ from Metcalfe's (1960) data (this Chapter: Table 2).

Eleusine coracana has been designated an "aspartate former" (Rathnam and Das 1975a), which complies with its XyMS+ typing (this Chapter: Table 1).

CHAPTER 4

METABOLITE TRANSPORT IN LEAVES OF C_4 PLANTS: SPECIFICATION AND SPECULATION.

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4.1 ABSTRACT

The biochemical and structural bases for intercellular transport of photosynthetic intermediates and products in leaves of C_4 plants are reviewed. The histochemical application of ribulose bisphosphate carboxylase antiserum to determine the source and sink relationships for C_4 acid transport in elaborate forms of leaf anatomy is described. Possible transport processes and pathways are discussed.

4.2 INTRODUCTION

Two major symplastic transport phenomena may be identified in leaves of C_4 plants. First, *intermediates* of photosynthesis move between two chlorenchymatous cell types, one of which is specialised for carbon assimilation and the other for carbon reduction. Second, *products* of photosynthesis move from the latter, or both cell types, to the phloem. Here, I review the biochemical and anatomical bases of these phenomena at the cellular level, emphasising major variations of C_4 leaf anatomy, introduce a new histochemical approach to these systems, and discuss the implications of structural variation for transport.

4.3 SPECIFICATION

4.3.1 Biochemical Bases

The C_4 pathway of photosynthesis is best defined in terms of radiotracer kinetic experiments, which demonstrate that C_4 dicarboxylic acids are both initial products and primary intermediates of $^{14}CO_2$ assimilation (Hatch 1976a). Studies on cellular localisation of C_4 photosynthetic enzymes, and on the complementary metabolic capacity of the two cell types isolated from leaves, clearly show that primary carboxylation of phosphoenolpyruvate is restricted to one cell type, with decarboxylation of C_4 acids and secondary carboxylation of ribulose-1,5-bisphosphate (RuP_2) restricted to the other (Black 1973; Hatch and Osmond 1976). I propose to denominate these cell types on the basis of function rather than histology. Cells having the capacity for C_4 acid synthesis I call primary carbon-assimilation (PCA) cells, and those with the capacity for carbon reduction via the photosynthetic carbon reduction cycle, PCR cells. Thus "mesophyll" cells of C_4 plant leaves correspond to PCA cells, and "chlorenchymatous bundle sheath", or Kranz cells correspond to PCR cells (see Chapter 1.2.).

Sources and sinks of photosynthetic *intermediates* may be specified at the intracellular level; e.g., for C_4 acids, the source is either the chloroplasts or cytosol of PCA cells, depending on whether malate or aspartate is transported to PCR cells. The sink is either the chloroplasts or mitochondria, depending on which of the three principal C_4 acid decarboxylase systems is found in PCR cells (Gutierrez *et al.* 1974a; Hatch *et al.* 1975).

The source of photosynthetic *products* has not yet been delimited. PCR, and PCA cells, are capable of sucrose synthesis (Downton and Hawker 1973; De Fekete and Vieweg 1973; Bucke and Oliver 1975), but if PCA cells synthesise sucrose *in vivo*, sugar phosphate precursors presumably are derived from PCR cells, increasing the intricacy of intercellular co-ordination and transport. I assume the phloem to be the sucrose sink.

4.3.2 Structural Bases

An essential starting point in the structural specification of the transport pathway for intermediates is the relationship between PCA and PCR cells, ideally identified on the basis of functional properties. Further, the transport of products demands detailed analysis of the pathways between PCR and vascular tissues. The location of PCR cells is central to both problems, and I have sought to identify these cells by fluorescent antibody labelling techniques (cf. Knox 1972/3).

I have used rabbit antiserum raised to purified RuP_2 carboxylase (anti-RuP_2Case) from wheat or spinach, to locate RuP_2Case in hand-cut leaf transections of both C_4 and C_3 species. Fixed material, treated with anti-RuP_2Case and fluorescein isothiocyanate (FITC) labelled anti-rabbit immunoglobulin, was examined in an epifluorescence microscope (see Chapter 5.3.). Fig. 7 shows leaf transections of *Triticum aestivum* (C_3), *Panicum maximum* (C_4), and *Pennisetum villosum* (C_4) (test serum, right; normal serum control, left). As expected, conspicuously FITC-fluorescing chloro-

plasts are found in all chlorenchymatous cells in wheat, but only in a sheath of cells (PCR) around the veins in the C_4 species. Weak fluorescence of PCA cell chloroplasts (cf. control), which persists when antiserum raised to the large subunit only of RuP_2Case is used, may be due to non-specific binding (see Chapter 5.4.2.B.).

The technique is particularly valuable for leaves not amenable to mechanical or enzymatic separation of PCA and PCR cells e.g., those of *Arundinella nepalensis* (Fig. 8). Here, longitudinal strands of cells are found, resembling sheath PCR cells, but isolated from the veins (Crookston and Moss 1973). Strand cell chloroplasts, as well as those of sheath cells, fluoresce markedly when treated with anti-RuP_2Case. Table 1 gives PCR cell locations in other C_4 species with unusual leaf anatomy, as detected by this technique.

Panicum milioides, which exhibits an "intermediate" CO_2 compensation point (Krenzer *et al.* 1975), was originally reported to have C_4 leaf anatomy (Downton 1971c). However, recent anatomical assessment suggests it is a C_3 plant (Chapter 2: Table 2); a view confirmed by physiological studies (Kanai and Kashiwagi 1975), $\delta^{13}C$ value (Brown and Brown 1975), and marked FITC-fluorescence of all chlorenchymatous cell chloroplasts (Fig. 16A). It will be interesting to apply fluorescent antibody labelling to other species claimed to represent intermediates between C_3 and C_4, and to artifically produced hybrids (Boynton *et al.* 1971).

It is now possible to pinpoint the PCA/PCR and PCR/vascular tissue interfaces in all types of C_4 leaves. For the latter interface, a major distinction may be drawn between two groups of C_4 grasses. The presence (XyMS+; e.g., in *Panicum maximum*, Fig. 7B) or absence (XyMS-; e.g., in *Pennisetum villosum*, Fig. 7C) of essentially chloroplast-free cells, in the mestome sheath position between metaxylem vessels and laterally adjacent PCR cells, seems to be correlated with the type of decarboxylase system in the PCR cells (Chapter 3).

Concerning PCA/PCR intercellular transport, it has been shown, in a quantitative assessment of the chlorenchyma of grass leaves, that no typical PCA cell is ever more than one other PCA cell from its nearest PCR cell (Chapter 2). Indeed, most PCA cells are in direct contact with PCR cells. Such relationships have not been examined in other families, and would be more difficult to assess in reticulately-veined leaves.

4.4 SPECULATION

4.4.1 Transport of Photosynthetic Intermediates

Measured rates of net carbon assimilation in C_4 plants imply rapid bidirectional movement of intermediates between PCA and PCR cells. The path taken is presumably symplastic, since plasmodesmata provide a lower resistance pathway than two plasmamembranes and a cell wall (Tyree 1970; Gunning and Robards 1976). Water flux, about 3×10^2 times greater than the metabolite exchange rate, is assumed to be apoplastic.

Two simple treatments of PCA/PCR symplastic metabolite exchange have been proposed. On one hand, plasmodesmata are regarded as open pores occupying approximately 2% of the PCA/PCR cell interface (Osmond 1971a). This estimate is supported by measurements of plasmodesmatal frequency and pore radii in *Salsola kali* (Oleson 1975) and *Zea mays* (O'Brien; pers. comm.). The pathlength for C_4 acid diffusion is taken as the difference between the mean radii† of the source (chloroplasts or cytosol of PCA cells) and sink (chloroplasts or mitochondria of PCR cells). In simple systems, with adjacent PCA and PCR cells, the pathlength ranges from about 15 μm in *Zea mays* to about 55 μm in *Amaranthus edulis* or *Atriplex spongiosa* (Osmond 1971a; Hatch and Osmond 1976). Even in a complex system (e.g., *Fimbristylis*: Table 1), the general pathlength is only 40 μm, with a maximum of 65 μm. However, two or more sets of plasmodesmata must be traversed.

On the other hand it is assumed that the transport pathway is

† "radii" being the distances from the centre of the vascular bundle.

restricted to plasmodesmatal desmotubules (which occupy only 0.1% of the PCA/PCR interface: Oleson 1975), that resistance to diffusion is restricted to the length of plasmodesmata, and that metabolites are perfectly mixed in the sources and sinks. This pathway would involve loading of a membrane system (endoplasmic reticulum?) contiguous with the desmotubule; a process currently defying speculation.

Osmond and Smith (1976) calculated that in either model, gradients of 10 mM between PCA and PCR cells would be sufficient to sustain diffusive metabolite fluxes compatible with net photosynthetic rates. Such gradients agree with independently derived estimates of the concentrations of C_4 intermediates (Osmond 1971a; Hatch and Osmond 1976). Hatch (1971) estimated that C_4 acid decarboxylation produces "total CO_2" concentrations of the order of 1 mM in PCR cells. The back flux of CO_2 to PCA cells may only be 10% of that of the metabolite flux (Hatch and Osmond 1976), and a tight stoichiometry between PCA and PCR cell reactions is probable.

Two implications of this discussion for C_4 plants with more complex leaf anatomy could be suggested. If the more tortuous transport pathway in species of genera such as *Aristida*, *Alloteropsis*, and especially *Fimbristylis*, limits metabolite transfer, we might predict larger pool sizes of photosynthetic intermediates than found in *Zea mays* for example. Alternatively, such species may photosynthesise at rates lower than normally found in C_4 plants.

4.4.2 Transport of Photosynthetic Products

Export of ^{14}C-labelled photosynthetic products and estimated total assimilate export are faster in leaves of C_4 plants than in C_3 plants (Hofstra and Nelson 1969; Lush and Evans 1974; Gallaher *et al.* 1975). Generally, the average pathlength between source and sink is shorter in C_4 plants, as reflected by lower interveinal distances (e.g. see Chonan 1972), and fewer plasmodesmata must be traversed, as indicated by lower lateral

cell counts (Crookston and Moss 1974; Chapter 2). C_4 plants do not necessarily have more phloem per leaf cross-section (Lush and Evans 1974; cf. Gallaher *et al.* 1975), but the key relationship of phloem area/ chlorenchyma area remains to be assessed. My speculations on sucrose transport will be confined to discussing likely intercellular pathways between chlorenchyma and phloem.

If PCA cells synthesise sucrose *in vivo*, the pathway of required sugar phosphate precursors, from PCR to PCA cells, would be the same as for other metabolites. Sucrose from PCA cells may take a similar path to PCR cells, *en route* to the phloem, since in all types of C_4 leaf anatomy, PCR cells lie between PCA cells and the veins. (An exception is the *Arundinella* type, where a minor proportion of PCR cells are embedded in PCA tissue). Such positioning means that if only PCR cells synthesise sucrose *in vivo*, the pathlength from source to sink is shorter than if PCA cells are also involved and thus still shorter than in C_3 plants.

There are several possible pathways for sucrose transport from PCR tissue to phloem. In leaves of XyMS+ C_4 plants (e.g., *Panicum maximum*: Fig. 7B), sucrose from adaxial PCR sheath cells may move tangentially, via cells in the mestome sheath (MS) position, to the cells abutting upon the phloem. Such a pathway is analagous to that suggested in wheat (Kuo *et al.* 1974). Two XyMS+ C_4 plants examined by Lush and Evans (1974) had lower phloem areas per leaf catchment area than two XyMS- plants also examined, suggesting that cells in the MS position may compensate for reduced phloem area. However, the XyMS distinction in C_4 grass leaves refers only to primary vascular bundles. In smaller bundles, as well as in *all* bundles of XyMS- plant leaves (Figs. 7C, 8), sucrose from adaxial PCR cells presumably moves tangentially through adjacent PCR cells to those abutting upon the phloem. Plasmodesmata occur in radial PCR cell walls of *Zea mays* (O'Brien and Carr 1970), but ignorance of plasmodesmatal frequency and distribution in PCR cells is substantial.

Transport of sucrose between PCR cells is also likely in species where many PCR cells are not associated with vascular tissue (e.g., *Triodia pungens*, *Salsola kali*: Table 1). In leaves where the PCR sheath is interrupted by metaxylem vessels (e.g., *Fimbristylis dichotoma*: Table 1),tangential transport from adaxial to abaxial PCR cells cannot occur, and the path may be through the sheath of essentially chloroplast free cells found external to the PCR sheath in such species. *Alloteropsis semialata* (Table 1) possesses a similar sheath, but the PCR sheath is complete.

Perhaps the greatest distances between PCR cells and phloem are encountered in C_4 leaf anatomy of the *Arundinella* type (Fig. 8). In *A. hirta*, there are no plasmodesmata between adjacent PCR cells of an isolated strand (Crookston and Moss 1973), and sucrose from these cells presumably moves through a symplast as extensive as that in some C_3 plants. In *Garnotia stricta* (Fig. 1B), there may be six such PCR strands between adjacent veins, with an interveinal distance up to 280 μm.

Comparison of assimilate translocation in different forms of C_4 leaf anatomy may help to specify which structural features influence rates of assimilate transport to phloem loading sites.

Table 1. Site of PCR cells in leaf blades of C_4 species exhibiting unusual C_4 leaf anatomy, as detected by immunofluorescent labelling of RuP_2Case in chloroplasts.

Species	*Location of PCR cells*
Gramineae:	
1). *Alloteropsis semialata*	innermost of the two recognisable sheaths surrounding the veins (cf. Ellis 1974b: C_4 form) (Figs. 12B, 12C).
2). *Aristida biglandulosa*	both recognisable sheaths surrounding the veins (cf. Brown 1975) (cf. *A. ramosa*, Fig. 13).
3). *Triodia pungens*	the sheath associated with veins and its extensions (Figs. 14A, 14B).
Cyperaceae:	
4). *Fimbristylis dichotoma*	the interrupted sheath, which lies between vascular tissue and the two recognisable complete sheaths (cf. Brown 1975) (Fig. 15B).
Chenopodiaceae:	
5). *Salsola kali*	innermost of the two chlorenchymatous cell layers which surround the entire leaf (cf. Oleson 1974; Carolin *et al.* 1975) (Fig. 14C).

CHAPTER 5

IN SITU IMMUNOFLUORESCENT LABELLING OF RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE IN C_3 AND C_4 PLANT LEAVES

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5.1 ABSTRACT

Antibodies raised to wheat and spinach ribulose-1,5-bisphosphate carboxylase (RuP_2Case) have been used to locate the enzyme in hand-cut leaf blade transections of 40 C_3 and C_4 species, and one Crassulacean Acid Metabolism (CAM) plant by immunofluorescence (using the "indirect" technique). The sample includes species from seven plant families, both monocotyledons and dicotyledons. In C_3 and CAM species, chloroplasts of all leaf chlorenchymatous cells immunofluoresce when labelled with anti-RuP_2Case, while in species with "classical" C_4 leaf anatomy RuP_2Case is located almost exclusively in "bundle sheath" ("Kranz" or PCR) cell chloroplasts. Ten C_4 species exhibit various types of "non-classical" C_4 leaf anatomy (*Alloteropsis*, *Aristida*, *Arundinella*, *Cyperus*, *Fimbristylis*, *Triodia*, and *Salsola* types), and for all but one of these types, immunofluorescent labelling of RuP_2 Case provides the first direct experimental evidence of a cellular compartmentation of photosynthetic carbon metabolism and of the location of PCR compartments. Leaves of two *Atriplex* C_3/C_4 hybrid individuals and of *Panicum milioides*, a C_3/C_4 intermediate, exhibited a C_3 antibody labelling response.

5.2 INTRODUCTION

Since the discovery of C_4-dicarboxylic acid photosynthesis, much effort has been directed to elucidating the detailed sequence of reactions in the pathway. A wealth of data indicates that these reactions are compartmented between and within cells with, for example, phosphoenol-pyruvate (PEP) carboxylation restricted to one chlorenchymatous cell type, while C_4 acid decarboxylation and ribulose-1,5-bisphosphate (RuP_2) carboxylation are restricted to another (see reviews: Hatch *et al.* 1971; Black 1973; Hatch 1976a; Hatch and Osmond 1976; also Usada *et al.* 1974; Huber and Edwards 1975a, 1975b; Ku and Edwards 1975; Rathnam and Das 1975b; Rathnam and Edwards 1975; Huber *et al.* 1976; and cf. Zelitch 1975).

The two cell types are usually designated "mesophyll" and "bundle sheath" respectively, and these terms adequately describe the sites of the compartments in plant leaves exhibiting "classical" C_4 ("Kranz") anatomy, i.e., that leaf anatomy first associated with C_4 photosynthesis (e.g. Downton and Tregunna 1968; see Laetsch 1974). Detailed experiments to locate individual enzymes of C_4 photosynthesis at the cellular level have been performed only on plants with this "classical" C_4 leaf anatomy, with the exception of two *Cyperus* species (Chen *et al.* 1973, 1974). The techniques used have all involved either tissue fractionation or isolation of organelles, protoplasts, or cells of the two compartments (reviewed Hatch and Osmond 1976). For critical results, procedures need to be well researched for each species (e.g. Huber and Edwards 1975c), and to date, fewer than thirty species have been investigated (references in Zelitch 1975; Hatch and Osmond 1976; plus Ku and Edwards 1975; Rathnam and Das 1975b). Moreover, for leaves of species exhibiting certain types of "non-classical" C_4 anatomy (e.g., spp. of *Aristida*, *Arundinella*, *Fimbristylis* and *Triodia*), it is questionable whether these procedures could be suitably adapted, and a different approach should be explored.

The sites of the two cell compartments in leaves with "non-classical" C_4 anatomy are often inadequately described by the terms "mesophyll" and "bundle sheath", and since so many C_4 species are now known where this is the case (see references Table 2) I prefer to call the two cell types "primary carbon-assimilation" (PCA) and "photosynthetic carbon-reduction" (PCR) respectively (Chapters 1.2 and 4.3.1.).

There is, as yet, no reliable histochemical means of recognising PCA and PCR cells. Staining for starch (Fig. 9A) is not always satisfactory since PCA cell chloroplasts contain starch under certain conditions (Fig. 9B) (Downton and Tregunna 1968; Hilliard and West 1970; Black and Mollenhauer 1971; Laetsch 1974; Forde *et al.* 1975; Shomer-Ilan *et al.* 1975; Rathnam *et al.* 1976). The only cellular components reported to be located exclusively in either PCA or PCR cells are certain enzymes of photosynthetic carbon metabolism (e.g. PEP carboxylase, pyruvate Pi dikinase, RuP_2 carboxylase). Immunofluorescent labelling of these enzymes (cf. Knox 1972/3) recommended itself as a likely means of identifying PCA and PCR cells *in situ*, being potentially applicable to all types of C_4 leaf anatomy. Such an approach, if successful, would allay, or substantiate, the doubt which continues to be expressed on the location of the above enzymes; e.g. RuP_2 carboxylase (see Zelitch 1975; Kelly *et al.* 1976). RuP_2 carboxylase seemed to offer the best chance of success for the following reasons:

1) The enzyme is an abundant leaf protein (e.g. see Kawashima and Wildman 1970; Gray and Kekwick 1974b) and purification procedures are well established; therefore, it is easily obtained for the purpose of immunisation.

2) Anti-RuP_2 carboxylase sera have been successfully raised by numerous workers (e.g. Gray and Kekwick 1974a; Nishimura and Akazawa 1974).

3) Successful *in situ* fixation and immunofluorescent labelling would presumably be favoured by the large size of native RuP_2Case, by its abundance, and by its confinement to chloroplasts.

4) Antiserum raised to RuP_2Case from one species is known to cross-react with enzyme from a wide variety of other species (e.g. Gray and Kekwick 1974a; McFadden and Tabita 1974; and see Gray and Wildman 1976).

I report the successful *in situ* immunofluorescent labelling of RuP_2Case in hand-cut leaf blade sections of 40 C_3 and C_4 species, of *Kalanchoë daigremontiana* (a Crassulacean Acid Metabolism plant), of *Panicum milioides*, and of two *Atriplex* C_3/C_4 hybrids, using antisera raised to wheat and spinach enzyme.

5.3 MATERIALS AND METHODS

5.3.1 Preparation of Ribulose-1,5-Bisphosphate Carboxylase

RuP_2Case (E.C.4.1.1.39) was purified from leaves of *Triticum aestivum* (C_3), following the techniques described by Paulsen and Lane (1966) and Lorimer *et al.* (1976). 500 gm of young leaf tissue were extracted in a total of 1 ℓ. of buffer (100 mM K-phosphate, pH 7.4, containing 10 mM EDTA and 1 mM DTT). The protein precipitating between 33% and 50% $(NH_4)_2SO_4$ was collected and resuspended in a minimum volume of extraction buffer. This solution was desalted on Sephadex G-25, then further fractionated with $(NH_4)_2\ SO_4$ (between 38-50%) and gradient-eluted from DEAE-cellulose with K-phosphate buffer (5 mM-250 mM, pH 7.6, containing 1 mM DTT). The partially purified protein was then concentrated with 60% $(NH_4)_2SO_4$ and applied to a 90 cm Sepharose 6B column equilibrated with 50 mM Tris buffer, pH 8, containing 1 mM EDTA and 1 mM DTT.

The protein peak corresponding to RuP_2Case was precipitated with 55% $(NH_4)_2SO_4$ and stored at -20°C. Protein concentration was determined spectrophotometrically. A second batch of wheat enzyme was prepared as above, but with finer $(NH_4)_2SO_4$ fractionations and pooling fewer DEAE-cellulose fractions.

Preparations of the enzyme were homogeneous as judged by disc-gel electrophoresis on 5% polyacrylamide gels (Davies 1964). With approx-

imately 100 µgm. protein applied to the gel, only a single slow-moving band was observed.

5.3.2 Preparation of Antisera

Required volumes of $(NH_4)_2SO_4$-RuP_2Case slurry were centrifuged at 4°C at 10,000 r.p.m. for 10 minutes. The pellets were resuspended in distilled water and each made up to 0.5 ml. Three month old female rabbits were injected subcutaneously in the "groins" and "armpits" (Boreham and Gill 1973) with the 0.5 ml. wheat antigen emulsified with 0.5 ml. Freund's complete adjuvant. The injection and bleeding schedules were as follows: (1) Day 0: inject 10 mg. antigen (batch 1 RuP_2Case); day 7: inject 20 mg. antigen; day 14: bleed 1 (A/S-1: rabbit 1; A/S-2: rabbit 2); day 19: inject 20 mg. antigen; day 27: bleed 2 (A/S-3: rabbit 1). (2) Day 0: inject 10 mg. antigen (batch 2 RuP_2Case); day 8: inject 10 mg. antigen; day 15: bleed 1 (A/S-4: rabbit 3). (3) Day 0: inject 1 mg. antigen (batch 2 RuP_2Case); day 8: inject 1 mg. antigen; day 15: bleed 1 (A/S-5: rabbit 4). Rabbits were bled from the marginal ear vein. Blood was allowed to clot at 37°C for two hours, kept at 4°C overnight to contract, and then centrifuged at 3,500 r.p.m. for 10 minutes. Gifts of antisera to *Spinacia oleracea* (C_3) leaf RuP_2Case (anti-native enzyme: A/S-6; anti-large subunit only: A/S-7), prepared according to Sugiyama *et al.* 1968, Nishimura *et al.* 1973, and Nishimura and Akazawa 1974, were supplied by Dr. T. Akazawa. Normal and pre-immunisation control sera were obtained from untreated rabbits. Serum specificity was analysed qualitatively by Ouchterlony double diffusion.

5.3.3 Ouchterlony Double Diffusion Analysis

Pre-punched agarose gels (MCl Biomedical) were rehydrated, then equilibrated in 50 mM Tris buffer, containing 1 mM DTT and 1 mM EDTA (pH 7.4 at 25°C). Undiluted sera (10 µl per well) were run against RuP_2 Case (prepared from wheat, spinach, and *Euglena*: 2.5 mg/ml. and 5 mg/ml.

in Tris buffer; 10 µl per well), and against a crude leaf mash of *Saccharum officinarum* (C_4) for 36-48 hours at room temperature in a water-saturated atmosphere.

5.3.4 Immunoassay of RuP_2Case and PEPCase Activities

The general avidity of anti-wheat RuP_2Case serum was investigated by assaying the activity of *Spinacia oleracea* leaf RuP_2Case. The effect of anti-RuP_2Case serum on *Zea mays* (C_4) leaf PEPCase activity was also tested; PEPCase was prepared by $(NH_4)_2SO_4$ fractionation and elution from DEAE cellulose (Ting and Osmond 1973a).

For the RuP_2Case assay, 0.12 units (50 µg. protein) of enzyme were incubated at 26°C with varying amounts of anti-RuP_2Case serum (A/S-3) for 30 minutes in activating buffer (100 mM Tris HCl, pH 7.9, containing 20 mM $MgCl_2$, 10 mM $NaHCO_3$, and 1 mM DTT) in a total volume of 0.2 ml. Following incubation, 10 µl. $NaH^{14}CO_3$ (1.85 µmoles; 1.85 µCi) were added, and the reaction initiated by addition of 10 µl. (0.2 µmole) of RuP_2. The reaction was stopped after 1 minute by addition of 0.2 ml. 6N HCOOH, and the reaction mix was dried on a hot plate. After addition of 1 ml. water, the acid-stable radioactivity was determined by counting in toluene/triton scintillant.

For the PEPCase assay, varying amounts of enzyme (0.02 - 0.2 units) were incubated at 30°C with 50 µl. anti-RuP_2Case serum (A/S-3) for 30 minutes in assay buffer (25 mM Bicine, pH 7.9, containing 5 mM $MgCl_2$ and 1 mM $NaHCO_3$). Following incubation, the reaction mixture was diluted with 2.6 ml. of assay buffer. Crystalline malate dehydrogenase (18 units) and NADH (0.3 µmoles) were added, and the spectrophotometric assay (3 ml. total volume) was initiated by the addition of PEP (3 µmoles).

In both assays, controls were run using volumes of normal serum equivalent to those of antiserum in the tests.

5.3.5 *In situ Immunofluorescent Labelling of RuP_2Case*

The indirect ("sandwich") labelling approach was adopted, the method being adapted from Knox (1971). Segments of fresh blade material (approx. 1 x 5 mm.) from young fully expanded leaves were fixed in 70% ethanol for 2 hours. Segments were held in Elder pith and transections, 10-15 μm. thick, were cut by hand with a clean razor blade. Sections were rinsed briefly in buffered saline (0.1 M K-phosphate in 0.2 M NaCl, pH 7.5), and transferred to a glass well containing 8-10 μl. of antiserum (neat, or diluted with buffered saline). After 1 hour of incubation, sections were rinsed for 20 minutes in three changes of buffered saline (3-5 ml. per wash) with thorough but gentle agitation, then transferred to 8-10 μl. of fluorescein isothiocyanate (FITC) labelled sheep anti-rabbit immunoglobulin (Wellcome Reagents Ltd., Beckenham, England: 1.88 mg. antibody protein/ml., diluted 1:4 with phosphate-buffered saline). Sections were incubated for 1 hour in the dark, then rinsed for a total period of 20 minutes with three changes of buffered saline, then rinsed and mounted in 50% glycerol (aq.) containing 1% (w/v) thymol. Slides were kept in the dark until observation.

For each antiserum-labelling test, two controls were run: 1) as above, using normal or pre-immunisation serum instead of antiserum (called "normal serum control"), and 2) fixed and sectioned as above, but mounted directly with no further treatment (called "autofluorescence control").

All procedures were performed at room temperature. Sections were transferred from well to well using fine sable hair brushes, one brush per serum treatment.

Mounted sections were observed with a Zeiss Photomicroscope III set up for epifluorescence, using an HBO 200 W/4 mercury vapour lamp and with two KP500 excitor filters, an Fl 500 reflector, and a B53 barrier filter in the vertical illuminator Type III RS. Sections were photographed within 24 hours of preparation.

For some species, the standard procedure gave a negative or weak labelling response; e.g. *Alloteropsis semialata*, *Aristida* spp., *Danthonia bipartita*, *Tidestromia oblongifolia*, *Triodia pungens*. Here, clear reactions only at the lateral edges of leaf blade transections (Fig. 9C) suggested incomplete fixation of leaf segments. Longer fixation times did not improve immunofluorescence, implying that rapidity of ethanol penetration into leaf tissue may be an important factor. For *T. pungens*, nicking the adaxial leaf epidermis may have enhanced the speed of ethanol penetration since this resulted in a clear reaction (Fig. 14A). Post-section fixation (5-20 minutes) instead of the standard pre-section fixation was also successful (for *A. semialata*, *Aristida* spp., and *T. oblongifolia*: Figs. 12B, 13A).

Some preliminary data were illustrated with black and white prints (Figs. 7, 8). However, black and white film proved generally unsuitable for recording results, since it fails to distinguish adequately between auto- and immunofluorescing chloroplasts. Permanent visual records could most satisfactorily be kept as colour transparencies from Kodak High Speed Ektachrome reversal film (EHB 135; tungsten light; ASA 125; stored at 4°C until one day before use). All frames were taken using the automatic exposure facilities of the Zeiss photomicroscope camera (using integrated measurement), and without the use of filters additional to those in the vertical illuminator.

5.4 RESULTS AND DISCUSSION

5.4.1 Specificity and Properties of Anti-RuP_2Case Sera

Results of the various analyses on the RuP_2Case preparations and anti-wheat RuP_2Case sera (viz. disc-gel electrophoresis, double immuno-diffusion, and immunoassay of RuP_2Case and PEPCase activities), suggest the antisera raised were specific for RuP_2Case. In double immunodiffusion, a single precipitin band was observed when anti-wheat RuP_2Case was run

against the wheat enzyme. All anti-wheat RuP_2Case sera (A/S 1 to 5), when tested against wheat RuP_2Case showed complete identity with each other, and also with anti-spinach RuP_2Case sera (A/S-6,7). Anti-wheat RuP_2Case serum (A/S-1) cross reacted strongly with RuP_2Case prepared from *Euglena* and *Spinacia oleracea* and with crude leaf extracts of *Saccharum officinarum*, giving reactions of partial identity against purified wheat RuP_2Case. Normal and pre-immunisation sera did not cross-react with wheat RuP_2Case.

In the immunoassay, spinach RuP_2Case relative activity (% control: Table 1) decreased progressively as anti-wheat RuP_2Case concentration was increased. Since Miziorko *et al.* (1974) report that native spinach PEPCase and RuP_2Case have similar molecular weights and "virtually identical mobility in gel electrophoresis", PEPCase activity was also assayed with the antiserum. Maize PEPCase activity was not suppressed (Table 1), even when the assay contained 368x more antiserum protein than PEPCase protein. This result suggests that the RuP_2Case preparations were not contaminated with PEPCase, or that antibodies to wheat PEPCase, if raised, cannot recognise maize PEPCase. (Leaf PEP carboxylases are known to exist in a variety of iso- and alloenzymic forms: Hatch *et al.* 1972; Ting and Osmond 1973a, 1973b). In either case, it seems unlikely that there will be any labelling of PEPCase in C_4 plant leaves when using the antiserum for immunofluorescence.

5.4.2 In situ Immunofluorescent Labelling of RuP_2Case

5.4.2.A. General Considerations

Leaves of 42 species and of two *Atriplex rosea* (C_4) x *A.patula* ssp. *hastata* (C_3) hybrid individuals have been examined. This sample includes: (1) C_3, C_4, and Crassulacean Acid Metabolism (CAM) species; (2) representatives of all three known C_4 types (e.g. cf. Gutierrez *et al.* 1974a: Tables 1 and 3); (3) members of seven plant families, both monocotyledonous

and dicotyledonous; and (4) representatives of eight of the eleven groups of Australasian grasses delimited by Watson and Clifford (1976) (exceptions: bambusoids, centostecoids, Stipeae).

Immunofluorescence is regarded here as fluorescence observed in the anti-RuP_2Case serum test run which is not observed in the normal serum control (Figs. 10A cf. 10B; 11A cf. 11B; 12B cf. 12C; 13A cf. 13B; 14A cf. 14B; 16A cf. 16B). Detection of immunofluorescence was not hindered by chloroplast autofluorescence (Figs. 10C, 11C, 16C), since this is not at or near the wavelength of FITC fluorescence. Autofluorescence of other leaf components is sometimes similar to FITC-fluorescence (e.g. cuticle and sclerenchyma cell walls of some grasses), but here the yellow-green fluorescence is present in both controls as well as in the tests (cf. Figs. 10A, 10B, 10C). There is often a difference in chloroplast fluorescence between the normal serum and autofluorescence controls (not illustrated), which might be ascribed to differential leaching of leaf compounds (e.g. chlorophyll) in the respective treatments.

I detected no difference in immunofluorescent labelling response using different anti-wheat RuP_2Case sera (A/S-1 to 5: undiluted), and even using anti-spinach native RuP_2Case (A/S-6) and anti-spinach large subunit RuP_2Case (A/S-7) (cf. Figs. 10A, 11A, 13A, 14A, 14C, 15A, 15B). Concurrent experiments with several species using different antisera are indicated in Table 2. Dilution of antisera in concurrent runs gave variable results. Thus, the labelling response was weak in *Entolasia stricta* at a dilution of 16x and in *Portulaca oleracea* at 40x. However, the response was still strong in *Aristida* species at a dilution of 32x, and in *Brachiaria foliosa* at 50x (Fig. 12A).

5.4.2.B. General Labelling Results

Of the species examined (in Table 2), 12 appear to be C_3, since (1) conspicuously FITC-fluorescing chloroplasts were observed in all leaf chlorenchymatous cells in test runs (e.g. Figs. 9C, 17B, 19A), and (ii) none of these species are known or suspected to be CAM plants. Five (single asterisk, Table 2) have in fact been recognised previously as C_3 on the basis of various non-anatomical criteria. The remaining seven species (including *Panicum milioides* which is discussed later) are all grasses, and were independently assessed to be C_3 using anatomical parameters (Chapter 2). One known CAM plant, *Kalanchoë daigremontiana* (Table 2) was investigated, and the labelling response indicated that RuP_2Case is present in all its chlorenchymatous cell chloroplasts as in C_3 plant leaves.

By contrast, leaves of the remaining 29 species investigated (Table 2) are characterised by restriction of conspicuously immunofluorescing chloroplasts to certain chlorenchymatous cells only (designated PCR cells). 17 of these (single and double asterisk, Table 2) have been classed previously as C_4 using non-anatomical criteria ($\delta^{13}C$ value, CO_2 compensation point, ^{14}C labelling results, etc.). The other 12 species, or species from the same genus, have been recognised as C_4 on anatomical grounds (e.g. see Brown 1975; Carolin *et al.* 1973; and Chapter 2).

Most C_4 species in my sample exhibit "classical" C_4 leaf anatomy (spp. marked 'C' in Table 2), and in these the PCR cells constitute a single bundle sheath which is juxtaposed to mesophyll (PCA) cells. Fig. 10A illustrates how bundle sheath cell chloroplasts of *Digitaria brownii* (XyMS-), in the anti-RuP_2Case test, fluoresce very markedly compared with chloroplasts of adjacent mesophyll cells; and compared with all cell chloroplasts in the normal serum (Fig. 10B) and autofluorescence (Fig. 10C) controls. Fig. 11 shows comparable labelling results for a XyMS+ species (*Sporobolus fimbriatus*).

For leaves of most C_4 species there was no difference in PCA cell chloroplast fluorescence between the anti-RuP_2Case test and the normal serum control (cf. Figs. 10A and 10B). In a number of species, notably *Bouteloua gracilis*, *Brachiaria foliosa*, *Buchloë dactyloides*, *Gomphrena celosioides*, and *Salsola kali* (Fig. 14C), some immunofluorescence was detectable in PCA cell chloroplasts, but this was weak relative to that in PCR cell chloroplasts. For *G. celosioides* it persisted using different fixation times (1 or 2 hours), different aged leaves (1st or 4th pair of leaves from shoot apex), and different antisera (A/S-3,4, or 7), but id did not persist in a control with FITC-labelled sheep anti-rabbit serum only. For *S. kali* too, use of different antisera (A/S-3,6, or 7) gave indistinguishable results. In *Brachiaria foliosa*, however, dilution of A/S-3 down to 50X progressively reduced PCA cell chloroplast immunofluorescence whilst retaining a strong reaction in PCR cell chloroplasts (Fig. 12A).

These observations may reflect the presence of some RuP_2Case in PCA cell chloroplasts of certain C_4 species, though the amount may be very small depending on the sensitivity of the labelling technique as applied. Two inherent limitations of antibody labelling, however, preclude definite conclusions on the significance of the slight PCA cell chloroplast immunofluorescence observed. These are concerned with the specificity of the antisera, and therefore of the labelling. Firstly, although my results (see Chapter 5.4.1.) give no indication that wheat RuP_2Case used for immunisation was impure, the presence of trace amounts of highly antigenic contaminants could have elicited the production of extraneous antibodies. Secondly, antibodies to the RuP_2Case molecule itself could be non-specific; i.e., they might react with heterologous antigens in the leaf tissue. Nevertheless, my results for species with "classical" C_4 leaf anatomy demonstrate that RuP_2Case is located almost exclusively in the bundle sheath cells. This finding is in accord with

the majority of studies concerned with cellular location of this enzyme in C_4 plant leaves (see Chapter 5.2.), including the seven species in my sample which have been subjected to tissue fractionation techniques (double asterisk spp. Table 2). The technique can be relied upon, therefore, to investigate RuP_2Case distribution in plant leaves of species with "non-classical" C_4 leaf anatomy and of C_3/C_4 intermediates and hybrids.

5.4.2.C. Labelling in Species with "Non-classical" C_4 Leaf Anatomy

Ten species listed in Table 2 (and designated "NC") exhibit some type of "non-classical" C_4 leaf anatomy. This sample includes representatives of all such known types, except the "Kochioid" and "Kranz-suaedoid" types (Carolin *et al.* 1975). Although the chlorenchymatous cells in leaves of such types have been identified as either "Kranz" (PCR) or "mesophyll" (PCA) (see references in Table 2), tissue separation has been reported for one of them only (two species of the *Cyperus* type: Chen *et al.* 1973, 1974). Immunofluorescent labelling of RuP_2Case, therefore, provides the first direct evidence of a cellular compartmentation of this enzyme in many of these types, and PCR cells can be identified readily (Figs. 12B, *Alloteropsis* type; 13A, *Aristida* type; 14A, *Triodia* type; 14C, *Salsola* type; 15A, *Cyperus* type; 15B, *Fimbristylis* type).

The existence of a variety of C_4 leaf anatomical types together with an easily applicable method of reliable PCR cell identification, provides a unique opportunity for assessing which anatomical features are essential to the operation of C_4 photosynthesis, and which are non-essential. For example, PCA and PCR cells are not always found in direct contact with each other; in the *Cyperus* (Fig. 15A), *Fimbristylis* (Fig. 15B), and *Alloteropsis* (Fig. 12C) types, they are separated by a layer of essentially chloroplast-free cells. Neither do PCR cells always constitute a *single* bundle sheath; in the *Aristida* type (Fig. 13), there are two PCR

sheaths. PCR cells are not always juxtaposed to vascular tissue; in the *Triodia* type (Figs. 14A, 14B) PCR cells may drape from one vascular bundle to another, to form what Brown (1975) refers to as "bundle sheath extensions"; in the *Arundinella* type (Fig. 8), isolated PCR cell strands are embedded in PCA tissue and lie parallel to the vascular bundles, but are seldom in contact with them; and in the *Salsola* type (Fig. 14C), vascular contact is not established with every cell of the peripheral PCR layer.

Considering the immunofluorescent labelling results, and other work (see references for "NC" species in Table 2, and Laetsch 1974), I conclude that consistent features of C_4 leaf anatomy include, in addition to the existence of two chlorenchymatous cell types:

1) PCR tissue-intercellular gas space contact (cf. PCA-gas space contact) and/or PCA-PCR contact is limited, because: intercellular spaces rarely, if ever, occur between PCR cells; PCR cell surface area to volume ratio is low cf. PCA cells (PCR cells are square or rectangular in paradermal view); PCR cells are arranged in some sort of layer, on one side only of which do gas spaces in contact with the atmosphere occur, exceptions being isolated PCR strands in the *Arundinella* type (Fig. 8). These features may be important in reducing rates of CO_2 diffusion from PCR tissue, which need to be small relative to rates of C_4 acid decarboxylation if the CO_2 concentration in PCR tissue is to be high.

2) PCA and PCR tissues are usually in direct contact; if not (e.g. *Cyperus* type, Fig. 15A), the distance between the two tissues is small (<10 μm). Such proximity may be essential in relation to rapid rates of flux of inter PCA-PCR metabolite transport.

3) PCA tissue is always positioned between PCR tissue and the atmosphere. This may be related to PEP carboxylation being the primary carboxylation in C_4 photosynthesis.

4) The ratio of PCA to PCR tissue (expressed on a volume basis - Chapter 7) is low, and quite consistent within a narrow range for given C_4 species types. An "anatomical stoichiometry" may well exist therefore, which reflects the stoichiometric link between rates of C_4 acid production in PCA cells and assimilation rates of CO_2, from C_4 acid decarboxylation, via the photosynthetic carbon reduction cycle in PCR cells. To support a defined biochemical stoichiometry, relative photochemical capacities of PCA and PCR tissue (and therefore relative chlorophyll content, chloroplast numbers, size, and function), would need to be in an appropriate balance.

5.4.2.D. Labelling in *Panicum milioides* and Hybrids of C_3 and C_4 *Atriplex* Species

Leaf anatomy superficially resembling that of the "classical" C_4 type has been reported for *Panicum milioides* (Kanai and Kashiwagi 1975) and *Mollugo verticillata* (Laetsch 1971; Kennedy and Laetsch 1974). Moreover, *P. milioides*, and two related species (*P. hians* and *P. laxum*), exhibit CO_2 compensation point (Γ) values between those typical for C_3 and C_4 plants, while the Γ of *M. verticillata* is C_3 (Krenzer *et al.* 1975; Goldstein *et al.* 1976). Investigation of other specific indicators for C_4 photosynthesis in *P. milioides* and other work, however, suggest that this species too is C_3 (Brown and Gracen 1972; Brown and Brown 1975; Wynn *et al.* 1973; Gallaher *et al.* 1975; Kanai and Kashiwagi 1975; Chapter 2). In particular, *P. milioides* (and *M. verticillata*) exhibits a $\delta^{13}C$ value typical for C_3 species (Brown and Brown 1975), implying that there is no major fixation of carbon via PEPCase and RuP_2Case, in series, as in C_4 plants (at least under normal conditions).

Since low Γ values are generally associated with C_4 photosynthesis, where RuP_2Case is cellularly compartmented, it was of interest to label leaves of *P. milioides* with anti-RuP_2Case serum. Immunofluorescence was detected in all chlorenchymatous cell chloroplasts (Fig. 16A; cf. normal serum and autofluorescence controls, Figs. 16B, 16C); i.e., the labelling

response is C_3. This result is supported by recent cell separation studies on *P. milioides* (Ku *et al.* 1976). Intercellular compartmentation of RuP_2Case *per se*, is not essential, therefore, in effecting a lowering of the CO_2 compensation point.

A further group of plants exhibiting intermediate Γ values are hybrids between *Atriplex rosea* (C_4) and *A. patula* ssp. *hastata* (C_3). The range of Γ values, and of leaf anatomy exhibited in the hybrids was broad, and there was no obvious correlation of CO_2 compensation point with the degree of similarity of leaf anatomy between the hybrid plant and the C_4 parent (Björkman*et al.* 1971). All hybrids investigated exhibited $\delta^{13}C$ values typical of C_3 plants (Hatch *et al.* 1972), and RuP_2Case activities of F_1 and F_2 hybrids were close to that of the C_3 parent (expressed on a leaf area or fresh weight basis: Björkman *et al.* 1971). Fig. 17C shows that all chlorenchymatous cell chloroplasts of an F_1 hybrid leaf immunofluoresce when labelled with anti-RuP_2Case serum, as in leaves of *A. hastata* (C_3: Fig. 17B) and in contrast to leaves of *A. rosea* (C_4: Fig. 17A).

The F_1 hybrid, and leaves of an F_3 hybrid which also exhibited a C_3 labelling response, possessed bundle sheath cells containing abundant chloroplasts, yet the $\delta^{13}C$ values of these individuals (F_1 = -24.5‰; F_3 = -27.2‰) are consistent with previous observations which suggest no significant flow of carbon through C_4 acids during photosynthesis. Whether RuP_2Case compartmentation is ever realised in hybrids could be determined by large scale screening using the immunofluorescent labelling technique, and it would be interesting to relate this to leaf anatomy, to $\delta^{13}C$ values, and to Γ values.

In C_3 plants, the Γ value is probably a reflection of the kinetic properties of RuP_2 carboxylase-oxygenase and of glycolate pathway activity (Badger *et al.* 1975). In C_4 plant leaves, where RuP_2Case is restricted to

PCR cells and where a PEP carboxylation-C_4 acid decarboxylation cycle is compartmented between PCA and PCR cells, I conclude that the low Γ value is a result of the kinetic properties and location of PCA cell PEP carboxylase such that the C_4 acid decarboxylase, the RuP_2Case, and the glycolate pathway of PCR cells are buffered from the atmosphere (Hatch and Osmond 1976; Chollett 1976).

Considering all available data for *P. milioides* and *Atriplex* hybrids, and especially results on the location of RuP_2Case, I propose two explanations which may account for "intermediate" CO_2 compensation points. First, and perhaps the less likely, is that the kinetic properties of RuP_2 carboxylase-oxygenase of these plants may deviate from those of other higher plants (e.g.,Badger *et al.* 1975), having a lower $K_m CO_2$, or higher $K_m O_2$, or both. Second, there could be some form of a compartmented PEP carboxylation - C_4 acid decarboxylation system which could serve to lower intercellular CO_2. In *P. milioides* for example, the chlorenchymatous bundle sheath cells could function in the same way as PCR cells of C_4 species, with the immediately adjacent (or all) mesophyll cells being similar to C_4 plant PCA cells but possessing, in addition, a fully operational photosynthetic carbon reduction cycle.

If the latter hypothesis is true, radiotracer kinetic experiments should reveal some transfer of ^{14}C from the C-4 carboxyl of C_4 dicarboxylic acids to 3-phosphoglycerate and carbohydrates. Limited experiments to date, for both *P. milioides* and *Atriplex* hybrids (Kanai and Kashiwagi 1975; Goldstein *et al.* 1976; Björkman *et al.* 1971), give no such indication; but they were not designed to critically test the coexistence of C_3 and C_4 photosynthetic carbon metabolism in the manner suggested. It is conceivable, for example, that the flow of carbon through C_4 acids could only become a significant proportion of the total carbon assimilated, at CO_2 concentrations just above the CO_2 compensation point. The existence of an *intercellularly compartmented* PEP carboxylation - C_4 acid decarboxy-

lation system in *P. milioides* has, to some extent, already been tested (Ku *et al.* 1976), and although there is evidence of some compartmentation of C_4 acid decarboxylating enzymes, the activities are considerably lower than in C_4 plant PCR cells. Detailed examination of location of all enzymes of the system has yet to be made, and further cell separation work or immunofluorescent labelling is required.

Table 1. Titration of purified spinach RuP_2 carboxylase and maize PEP carboxylase with anti-wheat RuP_2Case serum (A/S-3).

Enzyme	*Ratio* A_{280} $\frac{antiserum}{enzyme}$	*Relative Activity (% activity of control)*
RuP_2Case	3.6	91.7
"	14.4	80.3
"	36.1	55.9
PEPCase	36.8	120.0*
"	73.5	104.0
"	368.0	142.0*

*Controls without normal serum. All other controls <u>with</u> normal serum.

Table 2. Species examined by *in situ* immunofluorescent labelling of RuP_2Case, showing antiserum (A/S) used, designation as C_3, C_4 or CAM, and whether leaf anatomy, if C_4, is "classical" (C) or "non-classical" (NC).

Family and Species	*A/S number*[†]	*C_3, C_4 or CAM*	*C or NC*[††]
Gramineae			
Alloteropsis semialata (R.Br.) Hitch.	3,4,5	C_4*[2]	NC[14,18,19]
Amphibromus neesii Steud.	3	C_3	-
Aristida behriana F. Muell.	1,3	C_4	NC[14,15,21]
A. biglandulosa J.M. Black	1,3	C_4	
A. ramosa R.Br.	1,3,4	C_4	
Arundinella nepalensis Trin.	3	C_4	NC[14,15,17]
Bouteloua gracilis (Willd.) Lag.	3	C_4*[4]	C
Brachiara foliosa (R.Br.) Hughes	2,3	C_4	C
Buchloë dactyloides (Nutt.) Engelm.	3	C_4*[4]	C
Chloris gayana Kunth.	3	C_4**[11]	C
Danthonia bipartita F. Muell.	1	C_3	-
Digitaria brownii(Roem. et Schult.)Hughes	1,5	C_4	C
Entolasia stricta (R.Br.) Hughes	1	C_3	-
Microlaena stipoides (Labill.) R.Br.	4	C_3	-
Oryza sativa L.	4,5	C_3*[7]	-
Panicum antidotale Retz.	3	C_4*[4]	C
P. bulbosum R.Br.	3	C_4*[4]	C
P. maximum Jacq.	3	C_4**[10]	C
P. miliaceum L.	3	C_4**[9]	C
P. milioides Nees ex Trin.	1,3	C?***	-
P. pygmaeum R.Br.	1	C_3	-
Pennisetum typhoides(Burm.)Stapf & Hubb.	1,5	C_4*[4]	C
P. villosum R.Br.	3	C_4	C

/continued

Table 2. (cont'd).

Family and Species	*A/S number*†	*C_3, C_4 or CAM*	*C or NC*††
Poa sieberana Spreng.	1	C_3	-
Rhynchelytrum repens(Willd.) Hubb.	3	C_4	C
Sporobolus fimbriatus Nees	3	C_4**[11]	C
Thyridolepis mitchelliana (Nees) Blake	6	C_3*[3]	-
Triodia pungens R.Br.	1,3	C_4	NC[20,22]
Triticum aestivum L.	3	C_3*[5]	-
Zea mays L.	4	C_4**[13]	C
Cyperaceae			
Cyperus rutilans(Clarke)Maiden et Betche	1	C_4	NC[14,21]
C. sanguinolentus Vahl.	3,7	C_4	
Fimbristylis dichotoma (L.) Vahl.	3,6,7	C_4	
Amaranthaceae			
Gomphrena celosioides Mart.	3,4,7	C_4*[4]	C
Tidestromia oblongifolia Standley	4	C_4*[6]	C
Chenopodiaceae			
Atriplex rosea L.	1	C_4**[12]	C
A. spongiosa F. Muell.	3	C_4**[11]	C
A. hastata L.	3	C_3*[1]	-
Salsola kali L.	3,6,7	C_4*[4]	NC[14,16,23]
Atriplex hybrids‡ F_1	3	-	-
F_3	1	-	-
Crassulaceae			
Kalanchoë daigremontiana Ham. & Perr.	2	CAM****	-
Gentianaceae			
Nymphoides indica (L.) Kuntze	3	C_3*[8]	-
Portulacaceae			
Portulaca oleracea L.	1	C_4*[4]	C

/continued

† A/S-1 to 5, anti-wheat RuP_2Case; A/S-6, anti-spinach native RuP_2Case; A/S-7, anti-spinach large subunit RuP_2Case. (See Chapter 5.3.2.)

†† # after "NC" refers to literature describing leaf anatomy:
14. Brown 1975; 15. Carolin *et al.* 1973; 16. Carolin *et al.* 1975; 17. Crookston and Moss 1973; 18. Ellis 1974a; 19. Ellis 1974b; 20. Jacobs 1971; 21. Johnson and Brown 1973; 22. McWilliam and Mison 1974; 23. Oleson 1974.

* Species independently designated as C_3 or C_4 by other workers, on non-anatomical grounds; 1. Bender 1971; 2. Brown 1975; 3. Christie 1975; 4. Downton 1975 (review); 5. Hatch *et al.* 1967; 6. Phillpot and Troughton 1974; 7. Takeda and Fukuyama 1971; 8. Van Steveninck *et al.* 1972.

** C_4 species for which leaf tissue fractionation or separation work has shown a cellular compartmentation of RuP_2Case: 9. Edwards and Gutierrez 1972; 10. Gutierrez *et al.* 1974c; 11. Hatch *et al.* 1975; 12. Huang and Beevers 1972; 13. Kanai and Edwards 1973a.

*** See text. Source of *P. milioides*: Plant Introduction, C.S.I.R.O., Division of Tropical Agronomy, QLD., CPI 53933.

**** See Osmond (1976).

ŧ *Atriplex* hybrid individuals from *A. rosea* (♀) x *A. patula* ssp. *hastata* (♂); F_1 from seed of plant 7723 (♀) x plant 8112 (♂); F_3 from seed of F_2 plant 7746-12 x C_3 parent plant. (Seed from Dr. O. Björkman).

CHAPTER 6

THE ANATOMY OF GRASS LEAF SHEATHS IN RELATION TO PHOTOSYNTHETIC PATHWAY

6.1 ABSTRACT

The leaf sheath anatomy of 53 grass species, both C_3 and C_4, has been examined in transection with especial reference to chlorenchyma-bundle sheath-vascular tissue relations. Observations have been related to leaf blade anatomy and photosynthetic pathway. Leaf sheath and blade anatomy differ markedly in many respects, including the absence of "classical" C_4 ("Kranz") leaf anatomy in the leaf sheath of most C_4 plants. However, previously described predictive anatomical parameters (the "maximum lateral cell count" and the "maximum cells distant count") are still valid in C_4 leaf sheaths, suggesting that carbon is fixed via the same photosynthetic pathway in the sheath as in the blade. Preliminary experimental evidence, including immunofluorescent labelling of ribulose-1,5-bisphosphate carboxylase in sheath tissue, is in accord with this view. Brief consideration of leaf sheath bundle sheath anatomy in relation to associations and homologies of "photosynthetic carbon-reduction" (PCR = "Kranz") tissue emphasises the need for detailed leaf developmental work.

6.2 INTRODUCTION

The grass leaf is typically comprised of two distinct parts: a distal blade and a proximal sheath. The ligular region, including the adaxial ligule itself, identifies the blade-sheath junction at the point where the leaf ceases to be adpressed to the culm or developing shoot. The blade is usually the most conspicuous part of the leaf. Sheaths, however, show considerable inter- and intraspecific variation in prominence, which depends on (for example) whether the plant is tufted or stoloniferous, whether internodes are long or short, whether a vegetative or flowering culm or stolon is examined, and on the developmental stage of the leaf examined. Part or all of a particular sheath may be covered by one or more sheaths of older leaves. Nevertheless, on any one plant, some sheaths will be exposed directly to the light, and green, presumably photosynthesising, tissue may be abundant. Net photosynthesis has in fact been demonstrated in leaf sheaths of *Zea*, *Sorghum*, and *Oryza*, but at low rates (Thorne 1959; Sato and Tsuno 1975; Tsuno *et al.* 1975). In some species, leaf sheaths appear to constitute the bulk of leaf material (e.g., *Entolasia stricta*), while blades may be relatively much reduced on stolons (e.g., *Cynodon dactylon*).

The grass leaf blade has been the subject of extensive anatomical studies, and the wealth of data thus revealed (see especially Metcalfe 1960) has been extensively employed in taxonomy (e.g., Brown 1958; Tateoka 1960; Clifford and Goodall 1967; Clifford *et al.* 1969; Watson and Clifford 1976). Of especial interest, has been the recognition that certain characteristics of leaf anatomy are associated with C_4 photosynthesis, and the functional significance of these features has been widely speculated upon (e.g., Björkman *et al.* 1974; Laetsch 1974).

Descriptive information on the leaf sheath, by contrast, is very sparse indeed(Lommasson 1957; Kaufmann 1959; Metcalfe 1960; Blackman 1971; Chonan *et al.* 1974), being sufficient merely to indicate that sheaths may

be anatomically very different from their corresponding blades, and suggesting that they probably exhibit taxonomic variation on a similar scale. There is much incentive to acquire comparative data on grass leaf sheaths, as a contribution to understanding the basic anatomy of this important group of plants. Furthermore, the leaf sheath promises to provide another system in which to investigate structure/function relationships in the context of photosynthetic pathways, and the preliminary study reported here seems relevant to a search for constant (i.e., functionally essential?) anatomical accompaniments to C_4 photosynthesis (cf. Chapter 2). In addition, since little effort has yet been devoted to investigating the possibility that different parts of a plant may exhibit different photosynthetic pathways (but see Kortschak and Nickell 1970), it was desirable to compare some blades and sheaths in that respect.

6.3 MATERIALS AND METHODS

Young fully-expanded leaves of 53 grass species (39 genera: Table 1), from greenhouse or field-grown plants, were hand-sectioned transversely 1 cm below the ligule (sheath section) and, for comparison, one third the way up from the ligule (blade section). Temporary mounts in 50% glycerol (aq.:^{v}/v) were made of sheath and blade transections after staining in phenolic Bismarck Brown.

Low power stereoscopic examination in transmitted light reveals longitudinally consistent colour patterns of tissue organisation in the blades (see also Sutton 1973 for *Leptochloa fascicularis*, and Lush 1976 for *Panicum maximum*) and in the distal green zones of sheaths. However, the extent to which sheaths vary anatomically from distal (exposed, green) to proximal (enclosed, non-green) regions remains to be investigated.

The sample of 53 species includes C_3 and C_4 species, different C_4 type species (including *Panicum* spp.; cf. Table 1 and Gutierrez *et al.* 1974a, Table 1), examples of "non-classical" C_4 leaf anatomy and the

"Isachne-type" (Metcalfe 1960) C_3 leaf anatomy, and representatives from nine of eleven groups of grasses delimited by Watson and Clifford (1976).

For *in situ* immunofluorescent labelling of ribulose-1,5-bisphosphate carboxylase (RuP_2Case), leaf sheath sections were labelled as in Chapter 5.3.5.. CO_2 compensation points (Γ), for green leaf sheath tissue, were determined using a Maihak Unor 2 infra-red gas analyser linked to a constant-temperature leaf chamber (closed system).

6.4 OBSERVATIONS AND DISCUSSION

6.4.1 General Characteristics of Leaf Sheath Anatomy

Figs. 18, 19D, 20D, 21D, 22C, 23A, and 24B illustrate a representative range of leaf sheath anatomy, clearly showing that this differs markedly from blade anatomy (cf. Metcalfe 1960; Clifford and Watson 1976). General sheath characteristics in transection can be summarised as follows, with special emphasis on features in which they tend to differ from blades.

1) Chlorenchyma is generally restricted to the abaxial side of the sheath (Figs. 18, 19, 20, 21, 23B, 24B, 24C, 25B), and chloroplast density appears to be lower in these cells than in blade chlorenchyma. The chlorenchyma extends to a more or less constant depth, adaxially from the abaxial epidermis, in any given sheath transection (e.g., Figs. 18B, 19D, 21D, 22C, 23B). At the margins, where the leaf sheath is thinnest, chlorenchyma may constitute all the ground tissue between adaxial and abaxial epidermes.

2) Non-chlorenchymatous tissue is more extensive than in the blade (Figs. 20A, cf. 20D; 21A, cf. 21D; 22A, cf. 22B; 24A, cf. 24B) and is composed of "colourless cells" (see Metcalfe 1960). Such cells are highly vacuolated, sometimes contain a few cell chloroplasts (Figs. 18C, 24C, 25A), and usually have thicker walls than adjacent chlorenchymatous cells (Figs. 20D, 21D). They are mostly restricted to the adaxial part of the sheath, but often occur between the vascular bundles, and sometimes extend to the abaxial epidermis (Figs. 20D, 21D, 22B, 25B).

3) Lacunae are found in some sheaths, adaxially and between the vascular bundles. These lacunae may be very extensive: e.g., in and near the midrib area of *Oryza sativa* (Fig. 18A). Kaufmann (1959) has reported that lacuna formation in rice is a rhexigenous process, and I have observed torn cell walls bordering lacunae in most species which exhibit them: e.g., *Alloteropsis semialata* (Fig. 23C), *Amphibromus neesii* (Fig. 18B), *Dichanthelium clandestinum*.

4) Vascular bundles, which can always be clearly delimited, are situated abaxially (e.g.,Fig. 18), and when associated with sclerenchyma, the latter is predominantly found abaxial to the vein (Figs. 18B, 20D, 22B, 22C, 23B, 24B).

5) Stomata are rarely observed in the adaxial epidermis, in contrast to the abaxial epidermis (Figs. 18B, 20C, 23A). In any one sheath, they are more common in the lateral margins, where chlorenchyma may extend to the adaxial epidermis.

6) The adaxial epidermis of the sheath is not furrowed or ribbed as it often is in the blade (e.g., Figs 18B cf. 3C; 24B cf. 24A). Abaxially, however, furrows or ribs may be more prominent than in blade abaxial epidermis: e.g., in *Digitaria sanguinalis, Themeda australis, Triodia pungens* (Figs. 24C cf. 14B).

7) The leaf sheath is always thickest in the mid-vein region, tapering gradually towards its lateral margins.

8) Interveinal distances are generally greater in the sheath than in the blade (Table 2: but note *Leersia hexandra*).

6.4.2 C_3 and C_4 Leaf Sheath Anatomy

Additional to the general blade-sheath differences are others, specific to C_3 or C_4 plants, or to other interesting categories of grass species. For 16 out of 17 C_3 species, abaxial chlorenchyma in the leaf sheaths is continuous between adjacent vascular bundles (Figs. 18A, 18B, 19D), and the "maximum lateral cell count" (Chapter 2) is >4: i.e.,

as in C_3 blades. *Danthonia carphoides* sheath (Fig. 22B) alone was exceptional, with a lateral chlorenchymatous cell count of <4. In the blade, the count is also generally low (Fig. 22A), but it does exceed 4 between some vascular bundles. In both parts of the leaf, the "one cell distant criterion" is violated since the "maximum cells distant count" is >1 (a C_3 characteristic: Chapter 2). Nevertheless, *D. carphoides* sheath is further C_4-like in that colourless cells occur between the veins in contact with the abaxial epidermis (Fig. 22B); in this respect it requires further investigation, especially since its bundle sheath cells, unlike those of most C_3 species, clearly contain chloroplasts. *Panicum milioides*, currently of great interest as a putative C_3/C_4 intermediate (see Chapter 5.4.2.D), exhibits typical C_3 leaf sheath anatomy (Fig. 22C).

Leaf sheaths of five C_3 species which exhibit blade chlorenchyma of a distinctive kind were observed: viz., *Leersia hexandra*, *Oryza sativa*, and *Phragmites australis* whose blades possess "arm cells" (see Metcalfe 1960: and *Dichanthelium (Panicum) clandestinum* and *Isachne globosa* whose blades exhibit the "*Isachne* type" of leaf anatomy, characterised by radiate chlorenchyma consisting of narrow radially elongated cells (e.g. Tateoka 1956b, 1957; Metcalfe 1960) and observed in many C_3 eu-panicoid genera. In leaf sheaths, arm cells are still recognisable, although in *Leersia hexandra* the cell wall projections are less prominent. The "*Isachne* type" leaf anatomy, however, is not at all manifested in *D. clandestinum* and *I. globosa* leaf sheaths (Fig. 23A cf. 2C).

Out of 34 C_4 species examined, in only six was "classical" C_4 leaf anatomy clearly expressed in the leaf sheath: viz. *Bothriochloa macra*, *Echinochloa crus-galli*, and *Paspalum dilatatum* (but all with extensive adaxial colourless tissue and lacunae), and *Digitaria sanguinalis*, *Panicum bulbosum* and *Buchloë dactyloides* (but with many adaxial and interveinal "colourless cells": Figs. 20D, 21D). In most C_4 species, unlike C_3 plants, "colourless cells" intervene between laterally adjacent vascular bundles in the leaf sheaths, and although the total lateral cell count may be >4,

the count of "primary carbon-assimilation" (PCA: see Chapters 1.2 and 4.3.1) chlorenchymatous cells only is typically C_4: i.e., ≤4 (Figs. 20D, 21D). Chlorenchyma is, therefore, restricted laterally to the vicinity of vascular bundles, as well as abaxially as in C_3 leaf sheaths; and provided it is applied only to PCA cells (Chapter 2) the "maximum cells distant count" in C_4 plant leaf sheaths is one, just as in blades i.e., the "one cell distant criterion" holds. Furthermore, PCA tissue and "photosynthetic carbon-reduction" (PCR) tissue extend adaxially to similar depths in the sheath, such that they are always juxtaposed (Figs. 20D, 21D, 24B, 24C, 25B).

Of peculiar interest is the leaf sheath anatomy of species with "non-classical" C_4 leaf blade anatomy. That of *Alloteropsis semialata* (Fig. 23C) is, perhaps, more unlike the anatomy of its corresponding blade (Fig. 12B) than in any other species observed. The PCR sheath is still the inner of the two recognisable sheaths, as in the blade, but unlike PCR sheaths in leaf sheaths of other C_4 species, it contains chloroplasts even when not juxtaposed to clearly chlorenchymatous PCA (= "mesophyll") tissue. The PCR cell walls in *A. semialata* sheaths can be so thick, especially abaxially, that the lumen is nearly occluded, yet these cells still contain chloroplasts. In the blade, the outer bundle sheath is composed of cells which are small in diameter compared with inner bundle sheath (PCR) cells, and which contain few chloroposts. Outer bundle sheath cells in the leaf sheath, however, are larger in diameter than inner bundle sheath cells and can contain as many chloroplasts as surrounding PCA cells. The "maximum lateral cell count" in *A. semialata* (including the outer bundle sheath cells in the count) varies from three to eight (not illustrated) i.e., it is outside the limit for C_4 species (Chapter 2); and the "one cell distant criterion" is violated also. The distribution of PCA ("mesophyll") tissue, when apparent, is more C_3-like than C_4-like.

In *Aristida biglandulosa* (Fig. 24B) and *A. ramosa* leaf sheaths, the PCR sheath is double as in leaf blades (Fig. 24A) (see also Lommasson

1957); otherwise PCA-PCR relations are like those of other C_4 plants. *Arundinella nepalensis* blades (Fig. 8) are characterised by the occurrence of longitudinally-orientated strands of PCR cells, isolated from vascular tissue, between adjacent vascular bundles (see e.g., Crookston and Moss 1973). In the leaf sheaths, however, such strands are far less conspicuous (Fig. 23B) in transection, and in paradermal view are discontinuous and less extensive. Nevertheless, the "one cell distant criterion" is still valid.

A fourth type of "non-classical" leaf blade anatomy, the *Triodia* type (Fig. 14B), is again not manifested in the leaf sheath. In *Triodia pungens* sheath (Fig. 24C), PCR cell layers do not extend laterally from one vascular bundle to another, colourless cell tissue is predominantly adaxially distributed, and adaxial furrows, which are so prominent in the blade, are absent from the sheath. Abaxial furrows, however, are more prominent in the sheath than the blade. Stomata were observed only in the abaxial epidermis in the leaf sheath; in the blade, however, abaxial stomata are rare, occurring only where chlorenchyma is in contact with the abaxial epidermis. The leaf sheath anatomy of *T. pungens* is similar to that of other C_4 species, except that PCR sheaths abut onto abaxial epidermis and are never complete around the phloem end of vascular bundles.

Notwithstanding blade-sheath anatomical differences in C_4 plant leaves, the basic characteristics of C_4 leaf anatomy as outlined in Chapter 5.4.2.C appear to be consistent throughout green leaf material. The parameters used to distinguish C_3 and C_4 plant leaf blades (Chapter 2) are still applicable to the sheath (but see *Alloteropsis semialata*, above) despite greater interveinal distances in sheaths (Table 2) and a lack of "classical" C_4 leaf anatomy, and these results strengthen the suspicion that these parameters are functionally significant in grass leaves.

6.4.3 Photosynthetic Pathways in Blades and Sheaths

Anatomical evidence presented suggests that the path of carbon in photosynthetic carbon metabolism in the leaf sheath is the same as in the blade. Limited experimental data support this deduction, as follows:

i) responses to immunofluorescent labelling of RuP_2Case in leaf sheaths of seven species (C_3: *Amphibromus neesii*, *Panicum pygmaeum*; C_4: *Brachiaria foliosa*, *Buchloë dactyloides*, *Chloris gayana*, *Panicum bulbosum*, and *Pennisetum typhoides*) conform with results for the corresponding leaf blades (cf. Chapter 5.4.2.); viz. all chlorenchymatous cell chloroplasts immunofluoresce in the sheaths of C_3 species (Fig. 19B cf. 19A), and only PCR cell chloroplasts immunofluoresce in the sheaths of C_4 species (Figs. 20B and 21B).

ii) Leaf sheaths of three species exhibited CO_2 compensation point values similar to those of their blades. *Entolasia stricta* (C_3): blade 34 ppm., sheath 37 ppm.; *Arundinella nepalensis* (C_4): blade 4 ppm., sheath 4 ppm.; *Brachiaria foliosa* (C_4): blade 0 ppm., sheath 6 ppm..

More experimentation on leaf sheaths is needed, but complications may arise with some species because of the high proportion of "colourless cells" in these organs. Dark respiration rates and the possible existence of "dead" pools of such metabolites as C_4 dicarboxylic acids could render difficult the interpretation of CO_2 compensation point values and radiotracer kinetic data respectively.

6.4.4 Leaf Sheath Bundle Sheaths, and XyMS and C_4 Species Type

In addition to chlorenchymatous tissue relations, bundle sheath configurations were examined in leaf sheaths, being of interest: (i) to the XyMS character, which for the blade apparently distinguishes NADP-malic enzyme from PCK or NAD-malic enzyme type C_4 species (Chapter 3), and (ii) to Brown's (1975) hypothesis that the PCR sheath in some C_4 species is homologous to the "mestome bundle sheath".

The XyMS designations of species based on leaf sheaths (Table 1)

mostly agree with those previously made on the basis of blade anatomy (Chapter 3)(Figs. 20D cf. 20A; 21D cf. 21A), and I infer that a photosynthesising sheath conforms with the blade not only in photosynthetic pathway (C_3 or C_4: see above), but also in C_4 type. However, *Stenotaphrum secundatum* leaf sheaths sometimes exhibited a variable XyMS condition, constituting only the second such finding in eu-panicoid plant leaves (*Spinifex hirsutus* blades also are variable; see Chapter 3.6). Variable XyMS conditions were previously reported for leaf blades of some andropogonoid species (Chapter 3.4). Of these, only *Saccharum officinarum* was examined in the sheath (Fig. 25A), and here the XyMS criterion as defined cannot be applied, since all primary lateral vascular bundles were not associated with PCA tissue at the lateral level of the metaxylem vessels. Two bundle sheaths can, nevertheless, be recognised around these large veins in transection. One fits the description of a "mestome bundle sheath", in that it has thick cell walls and is composed of cells smaller than adjacent chlorenchymatous cells, while the other I presume to be a parenchyma bundle sheath. Sometimes another layer of cells may occur (Fig. 25A), external to metaxylem vessels and internal to the presumed "mestome bundle sheath", such a layer of cells having previously been encountered only in *Panicum decompositum* blades (Fig. 4A).

S. officinarum sheaths exhibit yet another interesting phenomenon in relation to bundle sheath tissue relations. Small vascular bundles with PCR tissue occur in some transections abaxially adjacent to the primary laterals (Fig. 25A), a situation previously unrecorded in grass leaves. It is conjectural, however, whether the PCR tissue of these small veins is continuous with the outer or inner bundle sheath of the adjacent primary lateral vascular bundles. Some transections of *Alloteropsis semialata* sheaths exhibit a comparable vascular bundle "pairing" arrangement (Fig. 23C), and here a chlorenchymatous bundle sheath (presumed PCR) can be continuous between the vascular bundle pairs, the

leaf sheath being XyMS-.

Leaf sheaths of C_3 species sampled are, like the blades (Chapter 3), all XyMS+. Cells in the "mestome (inner) bundle sheath" position in primary lateral vascular bundles, unlike those of other bundle sheaths, tend not to obviously merge into colourless cell tissue adaxially (Figs. 18A, 18B, 22B; but cf. 23A), and in this respect they resemble vascular tissue. By contrast, "parenchyma (outer) bundle sheath" cells tend to remain clearly discrete only when associated with chlorenchymatous mesophyll. Bundle sheath/colourless cell tissue relationships of large vascular bundles in C_4 XyMS+ species are like those of C_3 species. i.e., the PCR (outer) sheath blends with colourless cells adaxially, whereas an inner ("mestome"?) sheath does not (Figs. 21D, 24C). By contrast, in XyMS- species, where there are no cells in the "mestome bundle sheath" position between metaxylem vessels and adjacent PCR sheath cells in primary lateral vascular bundles, the PCR sheath does not tend to blend obviously with colourless cells, and it retains its integrity adaxially as a bundle sheath layer although the cells contain no chloroplasts if adjacent to colourless cell tissue (Figs. 20D, 23B, 23C). Integrity of bundle sheaths judged from transections alone, however, may be misleading. The apparent occurrence of an occasional double parenchyma bundle sheath in *Isachne globosa* leaf sheath (Fig. 23A) needs further confirmation for this reason; but in *Chloris gayana* sheath, the double nature of the PCR sheath cell layer, at least in some parts of some vascular bundle complexes, seems quite clear (Fig. 25B).

Contemplation of leaf sheath vascular bundle sheath configurations complicates discussion of bundle sheath homologies (cf. Brown 1975). In particular, the following questions remain unanswered:

1) C_4 species with two recognisable bundle sheaths in large vascular bundles often exhibit only one in smaller veins (e.g., Fig. 21A). Is the PCR sheath in these small vascular bundles homologous to a "mestome" or "parenchyma bundle sheath"?

2) If in certain eu-panicoid genera (mostly XyMS- species), the PCR ("Kranz") sheath is homologous to the "mestome bundle sheath", is the surrounding PCA (C_4 "mesophyll") tissue homologous to the "parenchyma bundle sheath" tissue? If so, has the "true" mesophyll ceased to develop?

3) What are the homologies of sheath tissue in vascular bundles exhibiting a variable XyMS condition? (i.e., XyMS+ on one side, and XyMS- on the other side, as found in leaf blades of some andropogonoid species (Fig. 5B) and *Spinifex hirsutus*, and in *Stenotaphrum secundatum* leaf sheaths).

4) In many species, both XyMS+ and XyMS-, cells resembling "mestome bundle sheath" cells often occur adjacent to phloem or xylem only. What are the homologies of such incomplete "mestome" sheaths? (Figs. 4C, 5A).

5) What is the relationship, if any, between different kinds of bundle sheath tissue, presence or absence of osmophilic layers (e.g., see Carolin *et al.* 1973), and presence or absence of suberin lamellae (e.g., see O'Brien and Carr 1970)?

Clearly, better tissue characterisation and detailed developmental studies are needed if more insight is to be gained into PCA/PCR homologies, into the differentiation of the complex PCA/PCR system, and into the evolution of C_4 photosynthesis.

Table 1. Grass species examined in leaf sheath transection. Species grouped according to Watson and Clifford (1976). C_3, C_4, and XyMS designations from leaf sheath anatomy made according to Chapters 2.3 and 3.3.. (+) or (-) indicates XyMS+ or XyMS- respectively for C_4 species.

$\underline{C_4}$

Andropogonoids: *Bothriochloa macra* (-), *Hemarthria uncinata* (-), *Saccharum officinarum**, *Sorghum bicolor* (-), *Themeda australis* (-), *Zea mays* (-).

Eu-panicoids: *Alloteropsis semialata* (-), *Brachiaria foliosa* (+), *Digitaria sanguinalis* (-), *Echinochloa crus-galli* (-), *Panicum antidotale* (-), *P. bulbosum* (-), *P. effusum* (+), *P. laevifolium* (+), *P. maximum* (+), *P. miliaceum* (+), *P. simile* (+), *Paspalum dilatatum* (-), *P. distichum* (-), *Pennisetum typhoides* (-), *P. villosum* (-), *Stenotaphrum secundatum***.

Chloridoids (Eragrostoids): *Bouteloua gracilis* (+), *Buchloë dactyloides* (+), *Chloris gayana* (+), *C. truncata* (+), *Cynodon dactylon* (+), *Eleusine coracana* (+), *E. indica* (+), *Eragrostis cilianensis* (+).

Aristideae: *Aristida biglandulosa* (-), *A. ramosa* (-).

Arundinoids: *Arundinella nepalensis* (-).

Danthonioids: *Triodia pungens* (+).

* XyMS condition cannot be ascertained. See text.

** Variable XyMS condition. See text.

/continued

Table 1. (cont'd.)

C_3/C_4 Intermediate (?)

Eu-panicoid: *Panicum milioides* (+).

C_3 (all XyMS+)

Eu-panicoid: *Dichanthelium* (= *Panicum*) *clandestinum, Entolasia stricta, Isachne globosa, Panicum pygmaeum.*
Arundinoid: *Arundo donax, Phragmites australis.*
Danthonioids: *Danthonia carphoides*†, *D. linkii* var. *fulva.*
Stipeae: *Oryzopsis miliaceum.*
Festucoids: *Amphibromus neesii, Avena sativa, Hordeum leporinum, Poa sieberana, Triticum aestivum.*
Oryzoids: *Leersia hexandra, Oryza sativa.*
"unclassified": *Ehrharta erecta, Microlaena stipoides.*

† but see text.

Table 2. Relative leaf blade-leaf sheath interveinal distances, expressed as a percentage of leaf blade interveinal distance. Interveinal distances (centre to centre) are averages for the number of vascular bundles examined, which is indicated after blade or sheath, being the number in a complete half transection, or less than this if in parenthesis.

C_3 species		Relative Interveinal Distance (%)
Isachne globosa	Blade 9 Sheath 19	290
Leersia hexandra	Blade 17 Sheath 19	93
Phragmites australis	Blade (6) Sheath (9)	122

C_4 species		Relative Interveinal Distance (%)
Aristida ramosa	Blade 9 Sheath 11	102
Chloris truncata	Blade 35 Sheath 31	151
Cynodon dactylon	Blade 17 Sheath 15	135
Panicum laevifolium	Blade (4) Sheath (3)	140
Sorghum bicolor	Blade (11) Sheath (3)	387

CHAPTER 7

A NOTE ON RELATIVE VOLUMES OF MESOPHYLL AND BUNDLE SHEATH TISSUES IN GRASS LEAVES

7.1 INTRODUCTION

In Chapter 3, attention was drawn to correlations between certain ultrastructural, anatomical and physiological features of C_4 grass leaf blades and C_4 species type, viz. NADP-ME, NAD-ME, and PCK types (Gutierrez *et al.* 1974a; Hatch *et al.* 1975). Parenchyma sheath (PS) cell shape (Brown 1974) is of particular interest in that it appears to be correlated to some extent with XyMS condition (Chapter 3), and, as Brown implies, might distinguish NADP-ME type from NAD-ME or PCK type C_4 species. The coincidence between C_4 type and XyMS type samples and Brown's PS cell shape sample is too small to be sure, however, and for two species tentatively classed as XyMS- from Metcalfe's (1960) descriptions, the correlation with cell shape does not hold (*Fingerhuthia africana, Latipes senegalensis*). Furthermore, there is some overlap in PS cell shape (expressed as the ratio of longitudinal length to radial width; Brown 1974) between XyMS- and XyMS+ C_4 species, so that it may not hold as an absolute predictor of C_4 type. Nevertheless, the general relationship between PS cell shape and C_4 type is no less interesting from an anatomical-functional viewpoint than is the XyMS character.

While making observations in relation to C_3-C_4 anatomical differences (Chapter 2), I noticed that "photosynthetic carbon-reduction" (PCR) cells (= "Kranz" = "chlorenchymatous bundle sheath": see Chapters 1.2, 4.3.1) in NAD-ME and PCK type C_4 species usually seemed larger in transectional area than adjacent "primary carbon-assimilation" (PCA) cells (= "mesophyll"). By contrast, this was rarely the case for NADP-ME type C_4 species, and I wondered whether C_4 species types are characterised by different radial dimensions of PCR cells, and if so, whether this reflected differences in PCR cell shape. A greater transectional area of PCR compared with PCA cells in NAD-ME and PCK type C_4 plants, could mean that relative PCA-PCR tissue areas (and therefore volumes) may differ between C_4 species types. In addition, mesophyll/bundle sheath tissue

ratios of C_3 species may differ from C_4 PCA/PCR ratios, since lateral cell counts and interveinal distances of C_3 and C_4 plant leaf blades differ (Chonan 1970, 1972; Takeda and Fukuyama 1971; Crookston and Moss 1974; Kanai and Kashiwagi 1975; Chapter 2). PCA/PCR ratios could be of significance in relation to the balanced co-ordination of cellularly compartmented biochemical reactions, in C_4 photosynthesis itself and in different types of C_4 photosynthesis.

7.2 OBSERVATIONS AND DISCUSSION

PCR cell radial dimensions exhibit considerable variation within leaf blade transections, and even within individual PCR bundle sheaths. Radial dimension is related, in part, to position of a PCR cell relative to other tissues: for example, adaxial and abaxial PCR cells, especially when associated with sclerenchyma, tend to be smaller in transectional area than lateral ones. A full appreciation of PCR cell dimensions requires detailed measurements and position records, and minimum and maximum radial dimensions only of PCR cells lateral to the vascular bundles are considered here. The sample of 23 grass species listed in Table 1 includes 14 biochemically typed C_4 species; the remaining 4 C_4 species I assume to be NADP-ME, NAD-ME, or PCK type on the basis of XyMS classification (Chapter 3).

The reported ranges for radial dimensions (Table 1) suggest that PCR cells of leaves of NADP-ME type species indeed tend to be smaller than those of NAD-ME or PCK type species (total ranges: NADP-ME, 8-30 μm, usually <25 μm; NAD-ME and PCK types, 15-50 μm, usually >25 μm). Of species with "non-classical" C_4 leaf anatomy (see Chapter 5.4.2.C), cells of isolated longitudinal PCR cell strands in *Arundinella nepalensis* have a diameter of approximately 25 μm; while leaf blades of *Aristida biglandulosa* exhibit two PCR sheaths, the inner sheath cell radial dimension being about 20 μm, with that of the outer sheath about 15 μm. The range in radial

dimensions of lateral parenchyma bundle sheath cells exhibited by four C_3 species and a putative C_3/C_4 intermediate (*Panicum milioides;* see Chapter 5.4.2.D), is similar to the total range for PCR cells of C_4 species.

Relative PCA/PCR (C_4) and mesophyll/bundle sheath (C_3) areas and volumes were determined by cutting out and weighing appropriate tissue areas from camera-lucida drawings of hand-cut leaf blade transections. "Colourless cells" (see Metcalfe 1960) and cells containing very few chloroplasts, were ignored when drawing the limits of PCA or mesophyll tissue. There are no obvious inter-PCR cell gas spaces, so reasonable estimates of PCR cell volume *per se* could be made. For PCA tissue, however, gas spaces (including substomatal spaces) are prominent, and no attempt was made to exclude them from the PCA area measured. The ratios given in Table 1 for 23 species, therefore, are ratios of PCA tissue (cells + intercellular gas spaces) to PCR tissue (cells only). Ratios were determined from the widths of transections indicated for each species in Table 1.

Despite the crude assessment of relative tissue volumes, species examined fall into four rather discrete categories. The four C_3 species do indeed exhibit high ratios, although *Panicum pygmaeum* overlaps the higher part of the C_4 range. NADP-ME type C_4 species exhibit lower ratios than C_3 species, and among them are *Arundinella nepalensis* and *Aristida biglandulosa*. For *Arundinella*, isolated strand cells were included in the PCR area measured (Table 1) since immunofluorescent labelling of RuP_2Case (Chapters 4.3.2, 5.4.2.C; Fig.8) and ultrastructure (Carolin *et al.* 1973; Crookston and Moss 1973) suggest that strand cells are functionally equivalent to PCR bundle sheath cells. Strand cells comprised about 20% of the total PCR tissue area sampled. Including strand cells as PCA tissue gives a ratio of 3.4, still within the NADP-ME C_4 species range. For *Aristida biglandulosa*, both bundle sheaths were counted as PCR tissue, immunofluorescent labelling having shown RuP_2Case in the chloroplasts of both (Chapter

5.4.2.C; Fig.13), and the PCA/PCR ratio thus derived is low (1.4), falling within the NAD-ME type C_4 species range. However, (1) chloroplasts in the outer bundle sheath appear less dense than in the inner bundle sheath or PCA cells (see also Bisalputra *et al.* 1969), and (2) detection of RuP_2Case in chloroplasts of both bundle sheaths does not necessarily mean that the sheaths are functionally identical (chloroplast ultrastructure and position differs: Bisalputra *et al.* 1969; Carolin *et al.* 1973; Johnson and Brown 1973). It may be significant, therefore, that a calculation based upon the inner bundle sheath only as PCR gives a ratio of 4.1 i.e., within the NADP-ME type C_4 species range. *Aristida purpurea* has in fact been recognised as NADP-ME type by Gutierrez *et al.* (1974a).

Panicum milioides, a possible C_3/C_4 intermediate, has a mesophyll/bundle sheath ratio lying in the PCA/PCR ratio range for NADP-ME type species, being the same as that of a C_4 species of the same genus (*P. bulbosum*) (Table 1). This result for *P. milioides* (XyMS+) adds further interest to this much-discussed species (see Chapter 5.4.2.D), being intermediate between the ranges for the two groups of XyMS+ plants (viz. C_3 and NAD-ME or PCK type C_4). Since my sample is small, it is not clear how discrete are NAD-ME and PCK type C_4 species in PCA/PCR ratios, but there is an obvious tendency for PCK type species to exhibit the lowest ratios of all those species examined (Table 1).

Taxonomically, the PCA/PCR ratio divides C_4 species quite neatly into eu-panicoids and chloridoids (eragrostoids) (cf. Watson and Clifford 1976). Ignoring *Arundinella* and *Aristida*, subfamilies seem better distinguished by PCA/PCR ratio than by C_4 type; but the remarkable exception of *Panicum maximum*, with a ratio of 1, suggests that the PCA/PCR ratio is of some functional significance and is not purely a taxonomic consideration.

The concept of an "anatomical stoichiometry" reflecting biochemical stoichiometry in C_4 plant leaf blades has already been raised (Chapters 2.4,

5.4.2.C). If it is valid, high PCA/PCR ratios would be unexpected in C_4 species, and indeed all but one C_4 species examined exhibit a ratio <3. For C_3 species, however, there is no obvious reason why mesophyll/bundle sheath ratios, or lateral cell counts, should not exceed C_4 plant values, as indeed they characteristically do (Table 1, and Chapter 2.4), nor does it seem incongruous for both parameters to occasionally fall within the C_4 range.

The PCA/PCR ratio classes for C_4 species could result from one, or a combination of factors contributing to tissue volumes, and these (e.g. PCA gas space volume, PCA cell volume, shapes and dimensions of PCR *and* PCA cells) require detailed consideration. Quantitative relationships of vascular tissue to chlorenchymatous tissue may be indirectly involved, and are further of interest in relation to assimilate transport and XyMS type. Despite the inadequacy of available data, the general correlation of PCR cell radial dimensions (and presumably PCR cell shape; cf. Brown 1974) with PCA/PCR ratios suggests that higher ratios are simply due to larger PCR cell radial dimensions, which in turn may reflect cells that are radially elongated (or at least isodiametric) rather than longitudinally elongated.

The broad differences in PCA/PCR ratio between C_4 species with different phosphoenolpyruvate carboxylation-C_4 acid decarboxylation systems may reflect differences in relative PCA-PCR space requirements for "accommodating" cell organelles. For example, the importation of "reducing power" into PCR cells as malate (see Hatch and Osmond 1976) may mean that relative PCA-PCR chloroplast sizes and/or numbers are larger in NADP-ME type species than in NAD-ME or PCK types. Furthermore, mitochondria are intimately involved in C_4 acid decarboxylation in NAD-ME type C_4 species (e.g., Kagawa and Hatch 1975; Rathnam and Edwards 1975) and these organelles are generally regarded as more numerous and/or more dense in PCR cells of NAD-ME (and PCK) plant leaves than in NADP-ME type C_4 plant

leaves (see references in Hatch *et al.* 1975). There may also be a requirement for a lower PCR cell surface area, per unit PCR cell volume, in PCK type relative to NAD-ME type, and NAD-ME type relative to NADP-ME type C_4 species. This could be significant to rates of PCA-PCR metabolite transport, and/or to rates of "leakage" of CO_2 from PCR cells into PCA cells or gas spaces, relative to rates of C_4 acid decarboxylation.

Table 1. Radial dimensions of PCR and bundle sheath cells, and PCA/PCR and mesophyll/bundle sheath area ratios in C_4 and C_3 plant leaf blade transections. Numbers in parenthesis after species refers to lateral width of transection sampled (mm.) followed by number of vascular bundles therein. Designation of C_4 type taken from Edwards *et al.* (1971), Huber *et al.* (1973), Gutierrez *et al.* (1974a), and Hatch *et al.*(1975);
n.a. = not applicable; - = not determined.

	Species		C_4 type	Radial dimension range of PCR (C_4) or bundle sheath (C_3) cells (μm)	PCA/PCR (C_4) or mesophyll/bundle sheath ratio (C_3)
C_3 (all XyMS+):	*Danthonia carphoides*	(1.34;11)	n.a.	6-13	11.4
	Festuca asperula	(0.82;3)	n.a.	10-15	9.3
	Oryza sativa	(0.68;3)	n.a.	10-23	6.6
	Panicum pygmaeum	(1.31;4)	n.a.	30-50	4.7
C_3/C_4 (XyMS+)	*Panicum milioides*	(1.35;8.5)	n.a.?	15-27	2.8
C_4 (XyMS-)	*Digitaria sanguinalis*	(1.75;18.5)	NADP-ME	12-20	4.7
	Bothriochloa macra	(0.45;6.5)	-	10-25	3.1
	Pennisetum typhoides	(1.41;17)	NADP-ME	8-15	3.0
	Panicum bulbosum	(0.7;7.5)	NADP-ME	10-20	2.9
	Arundinella nepalensis	1.39;11)	-	10-30	2.6
	Panicum antidotale	(1.07;13)	NADP-ME	10-18	2.4
	Echinochloa crus-galli	(1.59;14)	NADP-ME	8-15	2.1
	Aristida biglandulosa	(1.20;6)	-	15-20	1.4
C_4 (XyMS+)	*Brachiaria foliosa*	(2.09;10)	-	30-50	2.2
	Panicum miliaceum	(1.33;5.5)	NAD-ME	30-40	1.7
	Eragrostis cilianensis	(1.79;13)	NAD-ME	20-35	1.4
	Eleusine indica	(1.90;12)	NAD-ME	25-37	1.3
	Bouteloua gracilis	(0.95;6)	NAD-ME	25-33	1.3
	Buchloë dactyloides	(0.59;4.5)	NAD-ME	24-30	1.2
	Cynodon dactylon	(1.42;12.5)	NAD-ME	20-28	1.1
	Sporobolus fimbriatus	(1.37;12)	PCK	14-28	1.2
	Chloris gayana	(1.82;17)	PCK	15-30	1.1
	Panicum maximum	(1.97;13)	PCK	20-45	1.0

CHAPTER 8

C_4 LEAF ANATOMY IN RELATION TO C_4 PHOTOSYNTHESIS

8.1 INTRODUCTION

Laetsch (1974) provides the only review which attempts to relate C_4 leaf anatomy to function. In it, he tacitly accepts a model of C_4 photosynthesis for which there is very little evidence, and against which the evidence is voluminous (e.g., see references in Chapter 5.2). In particular, the model requires that RuP_2Case is located in "mesophyll" (PCA) cell chloroplasts. Furthermore, I do not share Laetsch's conclusion that "CO_2 fixation pathways and leaf anatomy of C_4 plants are not causally related", and this thesis endeavours to show that at least some consistent anatomical features are found in C_4 plant leaves, and that these appear to be related to C_4 photosynthesis in a meaningful way.

8.2 ANATOMY

The existence of two chlorenchymatous cell types in leaf blades of some plant species, usually distinguishable by staining for starch, has long been recognised (e.g., Schwendener 1890; Rhoades and Carvalho 1944). The correlation of C_4 photosynthesis with species exhibiting the two cell types was reviewed by Laetsch (1974), and since the time when this correlation was established (mid- and late 1960's), not even those workers who do not subscribe to the series formulation of C_4 photosynthesis by Hatch (1976a) (e.g., Laetsch himself), have disputed that chlorenchymatous "mesophyll" (PCA) and "bundle sheath" (PCR) cells have different functions in C_4 plant leaves.

In addition to the presence of PCA and PCR cells (Chapter 5), C_4 plant leaves appear to be characterised by at least four other anatomical features (briefly outlined in Chapter 5.4.2.C.). These are:

1) *PCR-intercellular gas space and PCA-PCR contact is limited*

   (a) PCR cells are arranged in some sort of layer, on one side only of which do gas spaces in contact with the atmosphere

occur. Exceptions to this general phenomenom are the isolated PCR strands in *Arundinella*-type C_4 leaf anatomy - see Fig. 8.

(b) Intercellular spaces between PCR cells do not occur. This rather obvious feature has seldom been remarked upon (Laetsch 1971; Hatch and Osmond 1976).

(c) Of the combined PCA and PCR surface area exposed to intercellular gas space, Edwards and Black (1971b) estimate that only 10% is PCR.

(d) PCR cells have a lower surface area/volume ratio than PCA cells. No absolute measurements have ever been made, but PCR cell shape is square or rectangular in paradermal view (Holm 1901; Laetsch 1971; Crookston and Moss 1973; Brown 1974). By contrast, PCA cells characteristically exhibit protuberances of some kind at least in one dimension (Chonan 1970, 1972; Laetsch 1971). Ideally, surface area/volume ratios need to be considered at the tissue level. Data on relative tissue volumes are sparse however (Chapter 7), and have yet to be related to exposed surface areas.

2) *PCA tissue is always positioned between PCR tissue and the atmosphere*

This characteristic is observable in any C_4 plant leaf, but has attracted little attention (Brown 1975).

3) *PCA and PCR tissues are closely associated*

This feature is reflected by the low "maximum cells distant count" in leaf blades (Chapter 2) and sheaths (Chapter 6), and by low interveinal distances (Chonan 1970, 1972; Takeda and Fukuyama 1971; Crookston and Moss 1974; Kanai and Kashiwagi 1975). When PCA and PCR tissues are not in direct contact (e.g., in the *Cyperus* and *Fimbristylis* anatomical types), the distance between them is <10 μm..

4) *PCA/PCR tissue volume ratio is low*

Evidence that the volumetric PCA/PCR ratio in C_4 plant leaves is

lower than the mesophyll/bundle sheath ratio of C_3 plants was presented in Chapter 7. Other parameters also reflect the low proportion of PCA to PCR tissue: viz. (a) low interveinal distances and "maximum cells distant counts" (see section 3 above) and low "maximum lateral cell counts" (Crookston and Moss 1974; Chapter 2), and (b) low PCA/PCR numerical cell ratios (Laetsch 1971, 1974). Laetsch, however, presents data for only three species, and cites these ratios in relation to relative PCA/PCR cell organelle concentrations.

The five characteristics above serve to define C_4 leaf anatomy; taken together they constitute the only available definition now that "non-classical" C_4 anatomical types are known to be fairly common. There is a need to place absolute quantitative limits on some of these criteria, and perhaps these will need to be group-specific, say at the family level. For example, the parameters used for grasses in Chapters 2 and 6 do not appear to hold outside the grasses. The "one cell distant criterion" does not hold for *Fimbristylis dichotoma* (Cyperaceae), or, apparently, for many C_4 dicotyledons; see Figures in Rathnam *et al.* 1976).

Most features of previous conceptions of C_4 leaf anatomy are now shown to be absent from many C_4 plant leaves, or have never been adequately demonstrated: viz,

(i) Chlorenchyma not radiate; or PCA and PCR tissues not arranged in two concentric layers (see Chapter 2 cf. Laetsch 1974).

(ii) No direct contact between PCA and PCR tissue (Laetsch 1971; Brown 1975; Chapters 4 and 5).

(iii) No direct contact between PCR and vascular tissue (Carolin *et al.* 1973, 1975; Björkman *et al.* 1974; Brown 1975; Shomer-Ilan *et al.* 1975; Chapters 4 and 5).

(iv) No absolute interveinal distance differences between C_3 and C_4 species. Pooling of results for C_3 and C_4 species

from five reports (see section 3 above) indicates considerable overlap. This parameter incorporates vascular tissue dimensions and, for some species, non-chlorenchymatous tissue also.

(v) Thick-walled PCR cells? This feature, though commonly cited as distinctive (Laetsch 1974; Brown 1975; Hatch and Osmond 1976) has never been substantiated quantitatively, nor has it been put into context (e.g., "thick-walled" relative to PCA cells?).

8.3 C_4 PHOTOSYNTHESIS

The functional significance of C_4 leaf anatomy in relation to C_4 photosynthesis cannot be discussed without considering the functional significance of the C_4 photosynthetic pathway itself. Results presented in this thesis make no contribution to understanding the latter, and I accept the concensus of opinion that recently seems to have been reached on this matter (see Black 1973; Björkman 1975, 1976; Chollett and Ogren 1975; Ogren 1975; Rathnam and Das 1975b; Chollett 1976; Hatch 1976a, 1976b; Hatch and Osmond 1976). That is, the prime feature of the C_4 pathway of photosynthesis is to facilitate the concentrating of CO_2 at the site of RuP_2Case, relative to intercellular CO_2 concentrations, such that (in normal air: 300 ppm. CO_2, 21% O_2); (i) RuP_2 carboxylation is less competitively inhibited by O_2 and/or the RuP_2 carboxylation/oxygenation ratio is increased (see Bowes *et al.* 1971; Chollett and Ogren 1975; Chollett 1976; Hatch 1976a; Hatch and Osmond 1976); and/or (ii) stomatal resistance can be greater per unit rate of net photosynthesis (see e.g., Akita and Moss 1973; Gifford 1974; Hatch 1976a).

Certain direct physiological consequences flow on from (i) and (ii) above:

(a) Maximal net photosynthetic rates, in normal air, are usually

higher in C_4 than in C_3 plant leaves (e.g., see Gifford 1974 for highest maximum rates recorded, expressed on leaf area basis; and see Björkman 1975).

(b) Photorespiratory CO_2 production via the glycolate pathway is reduced, or negated in normal air (Ogren 1975; Hatch and Osmond 1976), and this probably contributes to (a) above. As discussed later, the lack of CO_2 release from C_4 plant leaves into CO_2-free air, or their zero CO_2 compensation point, does not constitute *per se* experimental evidence for a lack, in normal air, of photorespiratory CO_2 production, unlike perhaps the lack of response of C_4 plant net photosynthesis to changing O_2 levels (1% to 21%: see Hatch *et al.* 1971; and Chollett 1973, 1976 with respect to supra-atmospheric O_2 levels).

(c) Water use efficiency is usually higher in C_4 than in C_3 plant leaves (Dobrenz *et al.* 1969; De Parcevaux 1973; and see ref.'s in Hatch *et al.* 1971; Hatch 1976a, 1976b).

The ability to function thus can be related to environmental factors. The ability of C_4 plants to utilise low leaf intercellular CO_2 concentrations (cf. C_3 plants: e.g., see Björkman 1975; Hatch 1976b) and/or to enhance net CO_2 fixation for any given intercellular CO_2 concentration (cf. C_3 plants), by permitting high stomatal resistances, may be advantageous (i) under conditions of low water availability (i.e., hot and/or arid conditions), and/or (ii) under conditions of high irradiance (Björkman *et al.* 1974; Hatch 1976a). As is well known (e.g., Hatch *et al.* 1971; Sankhla *et al.* 1975), C_4 plants tend to be tropical in distribution, but this is not absolute, and neither do tropical plants tend to be C_4 on a large scale (Hofstra *et al.* 1972; Mooney *et al.* 1973/4; Phillpot and Troughton 1974; Long *et al.* 1975). Perhaps, as Teeri and Stowe (1976) suggest, C_4 plants tend to be tropical because they are disadvantaged in cooler habitats (see also Björkman 1975).

A confusing issue has been whether the refixation or recycling of photorespiratory CO_2 occurs and if it does, of what significance it is to C_4 photophysiology. Firstly, it is necessary to note that C_4 plants have the potential for photorespiratory CO_2 production, and that glycolate pathway enzymes are primarily located in PCR cells (e.g., see Frederick and Newcomb 1971; Hilliard *et al.* 1971; Chollett 1976; Hatch and Osmond 1976). The lack of detectable photorespiration in C_4 plant leaves was ascribed by Forrester *et al.* (1966) to the efficient refixation of photorespiratory CO_2, whereas Moss (1966) considered that C_4 plants did not actually photorespire. The issue generally seems to have been approached as an either/or situation (Osmond 1971b; Osmond and Harris 1971; Liu and Black 1972) and remains to be posed as such (Hatch 1976a). Recent reports, referring to indirect evidence, tend to discount recycling as a reality (Chollett 1976; Hatch and Osmond 1976).

Questioning the reality of recycling needs to be (i) divorced from the questioning of the reality of photorespiratory CO_2 production, and (ii) looked at in two ways: firstly PCA→PCR recycling, and secondly intra-PCR recycling. At 300 ppm. CO_2, the lack of the Warburg effect for C_4 plant leaves strongly suggests that there is little, if any, photorespiratory CO_2 production (irrespective of the existence of a PCA CO_2-trapping system or not; net CO_2 fixation does not change from 21% to 1% O_2 conditions). The question of recycling of photorespiratory CO_2 would not appear to arise therefore, for normal air conditions. However, the question of recycling of CO_2 produced from C_4 acid decarboxylation, and which "leaks" from PCR tissue to PCA tissue, does arise. Whether or not there is such leakage, and whether or not the leaked CO_2 is recycled, is unknown, but there would seem to be no advantage in a "leaked" CO_2 molecule being involved in a PEP carboxylation reaction rather than an atmospheric CO_2 molecule. What is important, is that rates of C_4 acid flux into PCR cells should greatly exceed rates of CO_2 and HCO_3^- efflux (see Osmond and Smith 1976).

Similarly, even if significant photorespiratory CO_2 production occurred in a C_4 plant in normal air, it is difficult to see how *its* recycling, whether PCA-PCR or intra-PCR, could be of any advantage to a plant (cf. Raven 1972) in terms of conserving photorespired carbon. Once a CO_2 molecule is photorespired it is effectively "lost" to the plant and is presumably indistinguishable from an atmospheric CO_2 molecule. Energetically, the only advantage of recycling, if any, could be the utilisation of excess energy and reducing power; e.g., "bleeding off" electrons from the photosynthetic electron transport chain when these cannot be utilised in the Calvin Cycle for CO_2 fixation. This could be one function for the glycolate pathway itself: to produce CO_2 when this is not available from the atmosphere, in order that the Calvin Cycle can keep turning (Osmond and Björkman 1972). Under conditions of stomatal closure, where it seems likely that at least some PCA-PCR recycling occurs (see below), there is no need to have an effective anatomical arrangement to ensure refixation.

At the CO_2 compensation point in a C_4 plant (e.g., when the plant is in a CO_2-free atmosphere, or when stomata are closed), the rate of CO_2 fixed by RuP_2 carboxylation equals the rate of CO_2 production via photorespiratory processes (plus dark respiration). Intracellular CO_2 concentration in *PCR* cells is likely to be similar to that in chlorenchymatous cells in C_3 plant leaves. Intra-*PCA* cell CO_2 concentration, however, must be at or near zero in C_4 leaves, so that a PCR-PCA diffusion gradient exists. Some CO_2 and HCO_3^- efflux from PCR to PCA tissue must occur, unless the PCR tissue is apoplastically and symplastically sealed. There must at least be some CO_2 and HCO_3^- leakage via the symplasm. Recycling of this leaked CO_2 must occur, or else the Γ value of C_4 plant leaves would not be at or near zero. Rates of CO_2 refixation by PEP carboxylation must equal the rate of leakage. The CO_2 which is refixed may or may not be actual photorespired CO_2, which contributes to the PCR tissue CO_2 pool.

The rate of PCR→PCA leakage might not equal (but will not be greater than) the rate of photorespiratory CO_2 production, but the latter must equal rates of *intra*-PCR refixation (i.e., RuP_2 carboxylation) when the plant is at equilibrium at the CO_2 compensation point. It becomes clear that lack of CO_2 release into CO_2-free air, or a zero CO_2 compensation point, cannot mean that photorespiratory CO_2 production does not occur in CO_2-free air, and must mean that some PCA→PCR recycling occurs, unless PCR tissue is 100% "CO_2-tight". The observation that some C_4 species (apparently NAD-ME and PCK type) exhibit a CO_2 post-illumination burst (Bulley and Tregunna 1970; Downton 1970; Brown and Gracen 1972; Wynn *et al.* 1973) strongly suggests that PCR tissue, at least in these species, is not 100% physically CO_2-tight, if indeed the CO_2 released is from C_4 acid decarboxylation as suggested. Some C_4 leaf anatomical types may be more physically CO_2-tight than others: e.g., the *Cyperus* or *Fimbristylis* types cf. the "classical" type.

8.4 LEAF ANATOMY AND C_4 PHOTOSYNTHESIS

Component reactions of the PEP-carboxylation/C_4 acid decarboxylation cycle in plants with C_4 photosynthetic carbon metabolism are spatially compartmentalised. Leaves possess two chlorenchymatous cell types which fulfil the rôles of the compartments. The cell types can be distinguished in an unambiguous way by the immunofluorescent labelling of RuP_2Case (Chapter 5).

The four other anatomical features which characterise C_4 plant leaves, like the differentiation of chlorenchyma into PCA and PCR tissue, can also be envisaged as playing integral rôles in C_4 photosynthesis, but these rôles are less apparent and all require further investigation.

1) The unexceptional occurrence of PCA cells between PCR cells and the atmosphere would seem logical, since PCA cell PEP carboxylase is the

primary carboxylator: i.e., the "CO_2 harvesting antennae" (Black 1973) should be nearer the CO_2 source. The kinetic properties of PEPCase, especially its high affinity for CO_2 (low $K_m^{CO_2}$), are central to the physiology of C_4 photosynthesis, giving C_4 plants the ability to utilise CO_2 at low intercellular concentrations; hence the zero CO_2 compensation point of C_4 plant leaves (see Hatch 1976a).

Anatomically, however, one can *imagine* PCA-PCR arrangements where PCA cells are not always interposed directly between PCR cells and the atmosphere, and yet where access of atmospheric CO_2 to PCA cells is not hindered: e.g., a random arrangement of PCA-PCR cell pairs. No such arrangements are known, however, and the C_4 anatomical characteristic in question, therefore, may be significant in other ways.

It may be an indirect manifestation of other C_4 anatomical features: e.g., (see below), the need for PCR cells to have low PCR-gas space contact, and, perhaps, the need for PCR cells to be nearer vascular tissue than PCA cells. In addition, in an evolutionary sense, PCR cells would appear to have been developed and modified from tissues previously serving other functions: e.g., in most grasses, from a bundle sheath of some sort. It is possible then, that there may be differentiational or developmental "restrictions" on the site of PCA and PCR cells.

The occurrence of PCA cells between PCR cells and the atmosphere may facilitate (near) complete and *immediate* refixation of CO_2 which "leaks" from PCR tissue, whether it be photorespired or from C_4 acid decarboxylation. As noted above (Chapter 8.3), it is difficult to see how this phenomenom could be of advantage to a plant, and it may therefore be an inevitable consequence of C_4 leaf anatomy.

2) PCA-PCR proximity is probably important with respect to inter PCA-PCR metabolite transport, as suggested previously (see Hatch *et al.* 1971; Carolin *et al.* 1973), and as explored by Osmond (1971a), Oleson (1975), and Osmond and Smith (1976). The constancy of the "one cell distant

criterion" (Chapter 2) suggests that the number of plasmalemmas to be traversed could be important in intercellular transport rather than absolute distance. No data are available, however, to ascertain the relative importance of these two factors in relation, for example, to rates of C_4 acid flux (see Chapter 4). Biophysical aspects require detailed investigation, and C_4 plants, with their varied leaf anatomy, should provide interesting systems for research.

Inter PCA-PCR metabolite transport cannot be divorced from the remaining issues to be discussed in this Chapter. For example, the interposed layer of cells between PCA and PCR tissue in *Cyperus* and *Fimbristylis* types (see Chapters 3 and 4) may increase the diffusion pathway considerably, as Smith (1971) has suggested. However, it may also reduce CO_2 "leakage" from PCR cells. C_4 leaf anatomy may be something of a compromise with respect to two demands on the PCA-PCR interface: that of permitting rapid rates of flux of metabolites, and that of being a CO_2-tight barrier.

3) PCR cells are apparently shaped and organised so as to reduce PCR-gas space and PCA-PCR contact, and this may be an essential part of the CO_2 concentrating mechanism. Although C_4 leaf anatomy has been recognised as an essential component of this mechanism (e.g., Hatch and Osmond 1976), some simple anatomical features which appear to be involved (Chapter 8.2) have generally been overlooked. Other anatomical features which have been held to be significant have yet to be investigated adequately: viz. the rôles of PCR cell wall thickness and suberin lamellae in reducing (or eliminating?) rates of apoplastic CO_2 efflux (see Osmond and Smith 1976 for discussion). It may be that reduction of PCR-gas space and/or PCA-PCR contact *alone* is sufficient to reduce CO_2 leakage relative to C_4 acid influx such that measured net photosynthetic rates are accounted for. There may be no need for physical CO_2 barriers.

The suggestion that a suberin layer may be impermeable to CO_2 (Carolin *et al.* 1973; Chollett and Ogren 1975; Hatch and Osmond 1976) requires further investigation. In particular, there is no work which demonstrates that the osmophilic layer as observed (Carolin *et al.* 1973) in most PCR cells can confidently be assumed to be suberin (but see O'Brien and Carr 1970; O'Brien and Kuo 1975). These considerations are very important with respect to estimations of CO_2 or HCO_3^- efflux from PCR tissue (see Hatch and Osmond 1976), since they relate to whether the symplasm is the only path for CO_2 or HCO_3^- transport between PCA and PCR cells. Quantitative information on the bases and assumptions made by Osmond and Smith (1976) needs to be acquired. Here too, examination of a range of C_4 leaf anatomical types would presumably be instructive.

Little light can be thrown on the CO_2 tight PCR tissue question (or on recycling) by consideration of carbon isotope discrimination *per se*. Hatch and Osmond (1976) advance the argument that a "CO_2 tight space" must exist otherwise $\delta^{13}C$ values for C_4 species would be nearer those for C_3 species. This view can be questioned on several counts:

(a) In any of the several independent publications on $\delta^{13}C$ value for C_3 and C_4 species, the range of values for the two groups come very close to one another (see Table 1). It cannot necessarily be assumed that the intra-C_4 and intra-C_3 ranges encountered, and the proximity of the two ranges, are entirely due to the effects of environmental factors on the $\delta^{13}C$ value. General differences in rates of "CO_2 leakage" from PCR tissue in C_4 plants could account for some of the intra-C_4 group variation.

Table 1. Ranges of $\delta^{13}C$ values for C_3 and C_4 plants

Family	$\delta^{13}C$ value (‰) C_4 range	C_3 range	Source
Chenopodiaceae	-11 to -19	-23 to -30	Smith 1972
Cyperaceae	-8 to -16	-23 to -38	Lerman & Raynal 1972
Gramineae	-9.5 to -15.9	-22.7 to -32.3	Smith & Brown 1973
SEVERAL	-6 to -19	-24 to -34	Smith & Epstein 1971
SEVERAL	-10 to -20	-22 to -33	Bender 1971
SEVERAL	-10 to -19	-21 to -36	Troughton *et al.* 1974
SEVERAL	-9.6 to -16.5	-21.2 to -31.7	Smith & Turner 1975

(b) The only calculations I have seen which relate $\delta^{13}C$ values to enzymic carbon isotope discrimination (Whelan *et al.* 1973) contain significant errors. In estimating the *in vivo* fractionation during PEP carboxylation they (a) ignore the $\delta^{13}C$ value of atmospheric CO_2, and (b) they correct for the Δ value between HCO_3^- and CO_2 in the wrong direction. Recalculated $\delta^{13}C$ values of C_4 acids are higher than Whelan *et al.*'s estimates, and are higher than for C_4 plant carbon as a whole, suggesting perhaps that some fractionation in RuP_2 carboxylation could occur. Furthermore, the contribution to the $\delta^{13}C$ value of a plant made by physical fractionation during diffusion of CO_2 into the leaf may have been mistakenly ignored.

(c) If CO_2 leakage from PCR tissue does occur (to varying degrees), PCA cell PEPCase could act as a trap to the escape of ^{13}C discriminated against by RuP_2Case. Presumably it is some combination of a CO_2-tight PCR tissue and a PCA trapping system which accounts for the apparent lack of discrimination against ^{13}C by RuP_2Case in C_4 plants.

4) "Anatomical ratios" of PCA to PCR tissue (e.g., volumetric ratio: Chapter 7) may reflect the (strict?) stoichiometry between the two compartments of C_4 photosynthetic metabolism i.e., these ratios may reflect tissue capacity to house the organelles required by each tissue type. Volumetric PCA/PCR ratio estimation is a first, crude attempt to assess such a relationship, and the results raise some interesting ideas: viz. (i) can PCA/PCR ratios be related to photochemical activity of PCA and PCR cell chloroplasts, and to differences in this respect between different C_4 types (e.g., related to work of Smillie, Boardman and co-workers in Hatch *et al.* 1971, and of Edwards *et al.* 1976)?; (ii) are PCR cell shape, PCR radial dimension, and PCA/PCR volume ratios related to rates of CO_2 leakage, and therefore to CO_2 concentrating mechanism? If they are, what is the significance of different C_4 species types, and of PCR cell chloroplast locations? Biometric relationships of PCA and PCR tissues need to be examined in detail considering, for example, organelle size and concentration, and cell numbers.

Anatomical features of C_4 leaf anatomy discussed here do not include the proximity of chlorenchyma to vascular tissue and its possible relationship to rapid rates of translocation of photosynthate from C_4 plant leaves (Downton and Tregunna 1968; Downton 1971d; Monteny 1973; Crookston and Moss 1974; Laetsch 1974; Lush 1976; Stephenson *et al.* 1976). This matter requires further investigation, including studies of plasmodesmatal frequency and distribution along the lines of Kuo *et al.'s* (1974) work. It would be interesting if differences in translocation rates and vein loading were to be discovered between different C_4 anatomical types.

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Fig. 1.

FIG. 1. C_4 species. Transections of grass leaf blades; adaxial epidermis uppermost. Prepared as in Chapter 2.3.. *A*, *Cynodon dactylon* (chloridoid), to illustrate typical "Kranz" anatomy (= "classical" C_4 leaf anatomy). Note radiate mesophyll and large PBS cells containing abundant chloroplasts. Lateral cell count 2 ("colourless cells" ignored). One cell violates the "one cell distant criterion" (arrowed; see text); X490. *B*, *Garnotia stricta* (arundinoid, XyMS- cf. Fig. 4C), mesophyll not radiate, arrows indicate "circular cells" of which there are six between the two vascular bundles. "Circular cells" = PBS cell equivalents = isolated PCR strands in transection; X330. *C*, *Stenotaphrum secundatum* (eu-panicoid), showing indistinctly radiate mesophyll and PBS cells smaller in transectional area than mesophyll cells. Lateral cell count 1-2; X350. *D*, *Setaria geniculata* (eu-panicoid), showing PBS cells smaller in transectional area than mesophyll cells, but containing abundant chloroplasts. Lateral cell count 2; X400. *E*, *Pennisetum villosum* (eu-panicoid), to illustrate one large atypical mesophyll cell violating the "one cell distant criterion" (arrowed). Lateral cell count 2-4. Darkground illumination; X235.

cc, "colourless cells"; ↔, line along which lateral cell count is made.

Fig.1

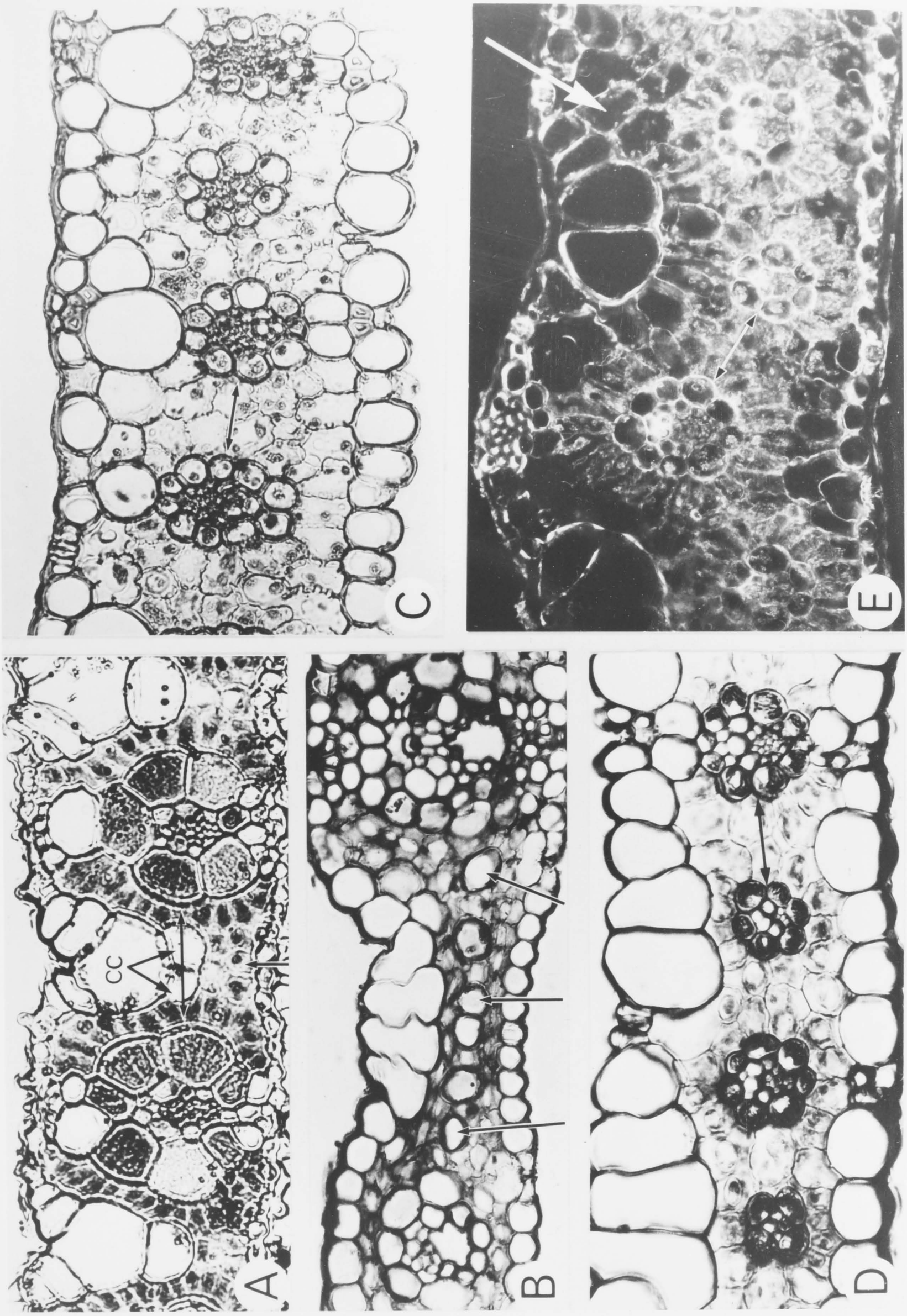


FIG. 2. C_3 species. Transections of grass leaf blades; adaxial epidermis uppermost. In each transection, mesophyll cells violate the "one cell distant criterion". Prepared as in Chapter 2.3.. *A, Arundo donax* (arundinoid), showing indistinctly radiate mesophyll, and PBS cells much larger in transectional area than mesophyll cells. The lateral cell count of 3 seen here is not maximal ("colourless cells" ignored); X225. *B, Catapodium rigidum* (festucoid), showing indistinctly radiate mesophyll. Lateral cell count >4; X430. *C, Entolasia stricta* (eu-panicoid), showing radiate mesophyll. Lateral cell count >4; X260. *D, Phragmites australis* (arundinoid), showing PBS cells containing abundant chloroplasts and larger in transectional area than mesophyll cells. Lateral cell count ≥4, not maximal; X225.

cc, "colourless cells"; ↔, line along which lateral cell count is made.

Fig.2

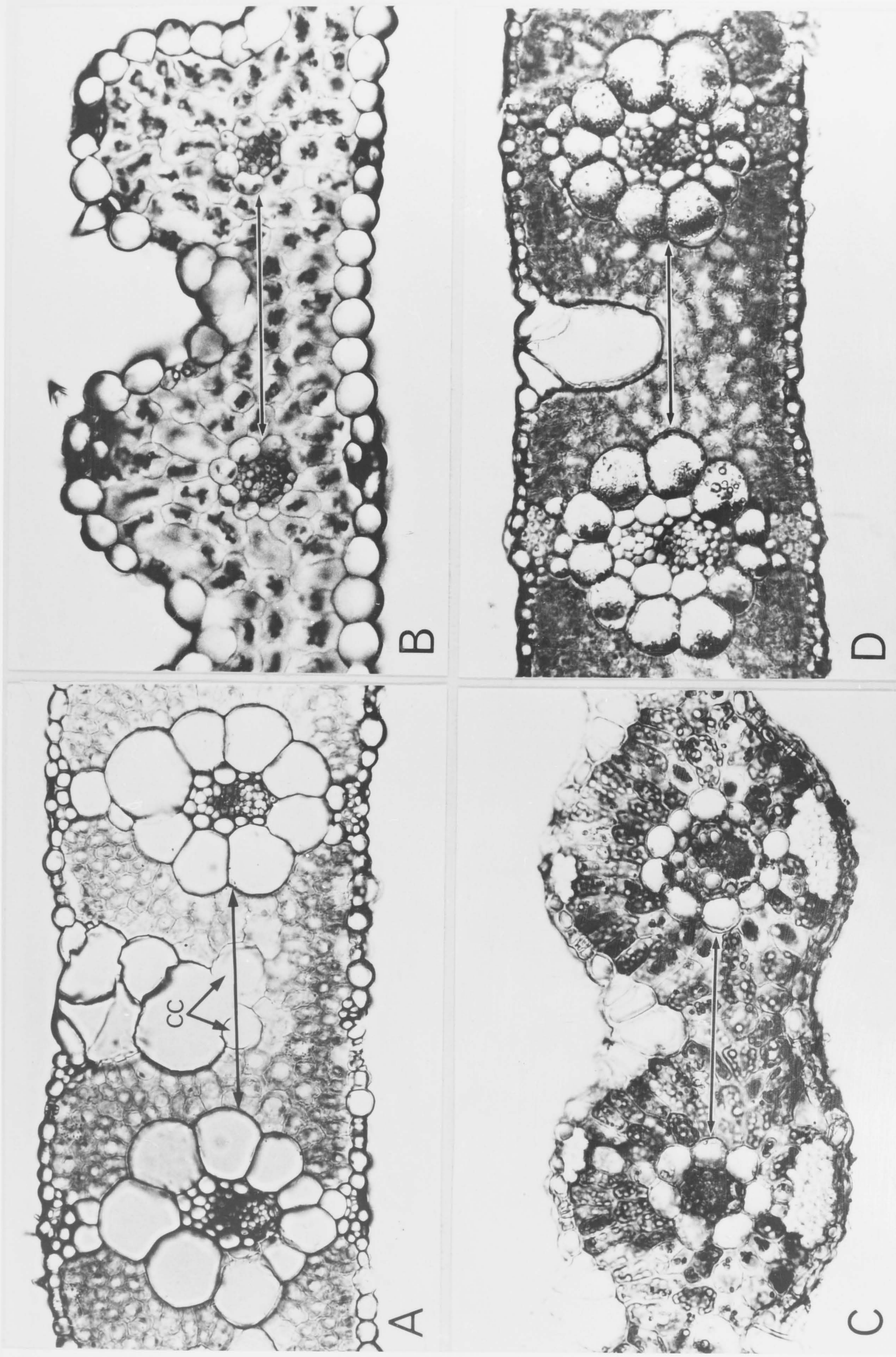


Fig. 3.

FIG. 3. C_3 species. Transections of grass leaf blades; adaxial epidermis uppermost. In each transection, mesophyll cells violate the "one cell distant criterion". All are autofluorescence micrographs prepared as in Chapter 5.3.5. for autofluorescence controls. *A*, *Thyridolepis mitchelliana* (eu-panicoid), showing clearly radiate mesophyll; X410. *B*, *Hordeum vulgare* (festucoid), showing abundant chloroplasts in PBS cells (arrowed); X255. *C*, *Amphibromus neesii* (festucoid: XyMS+, cf. Fig. 4B), showing an ill-defined PBS, especially abaxially. Note conspicuous adaxial ribbing, and that the "one cell distant criterion" is violated in the "domes" of these ribs as well as between the vascular bundles (arrowed); X410.

Fig.3

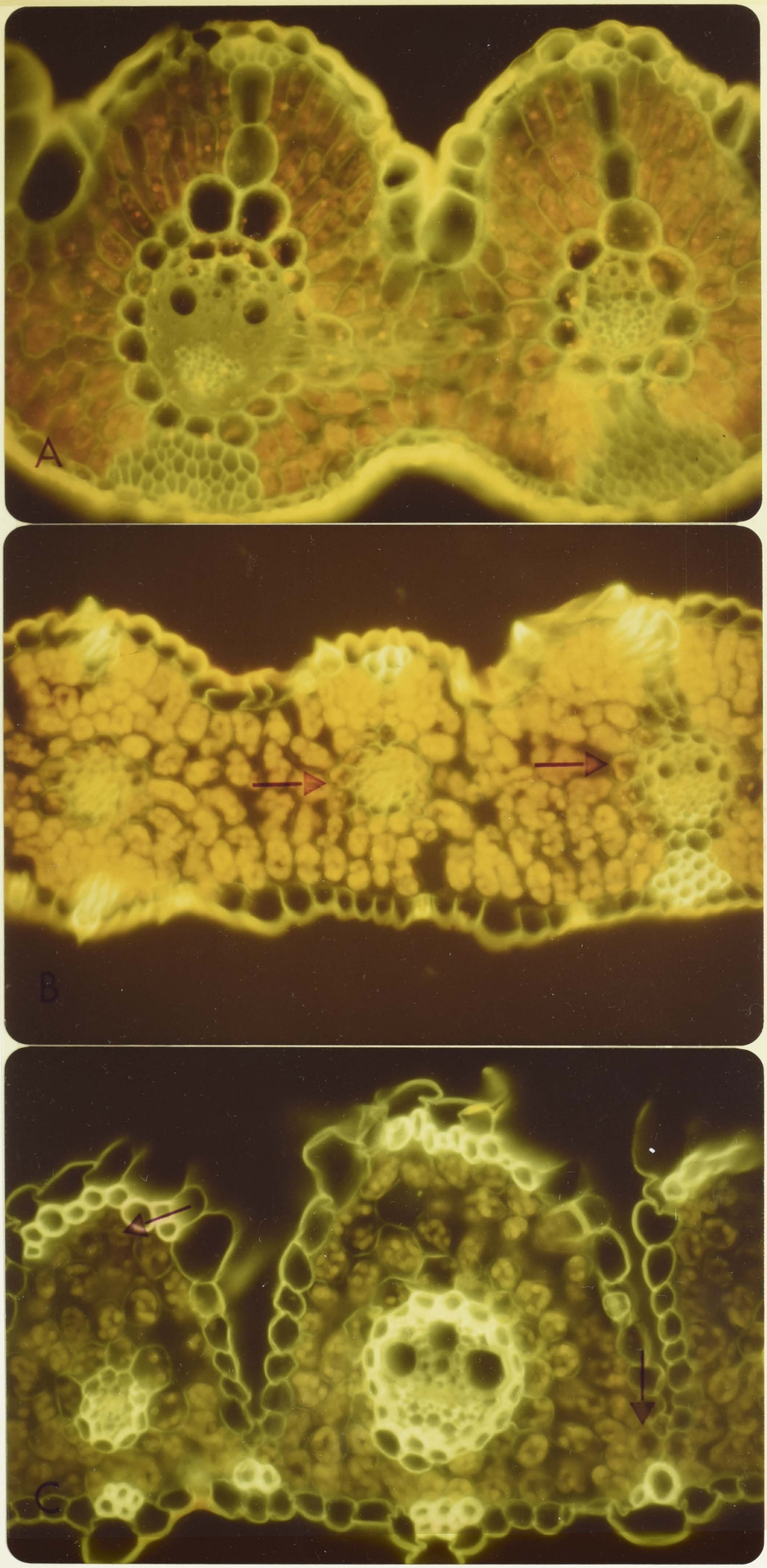
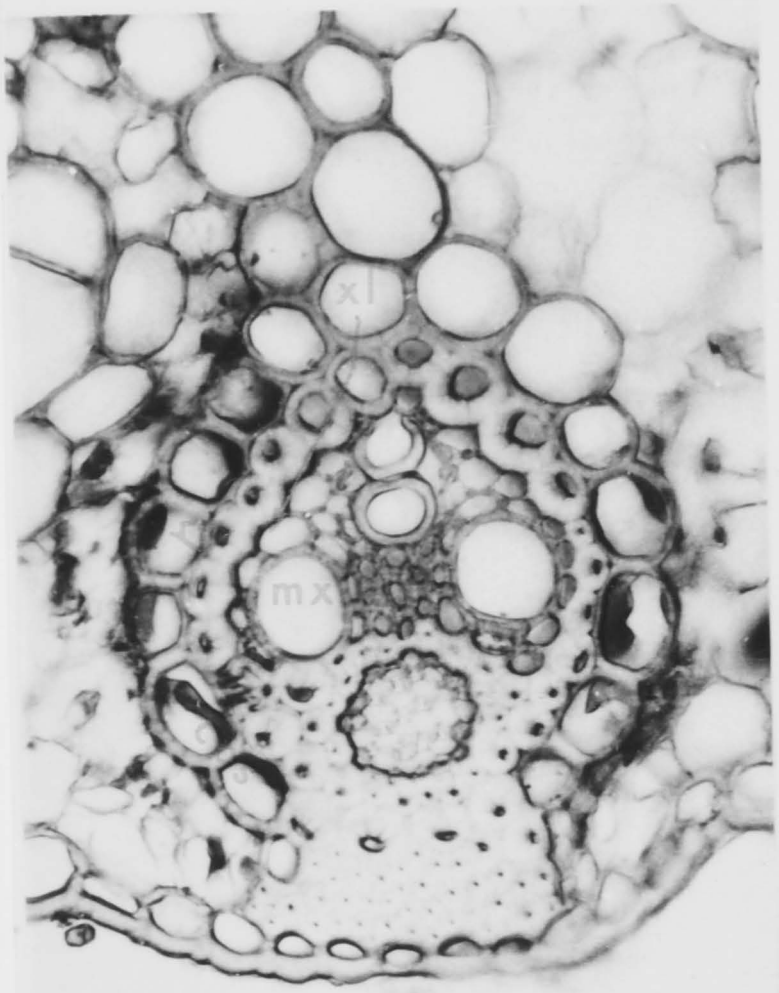


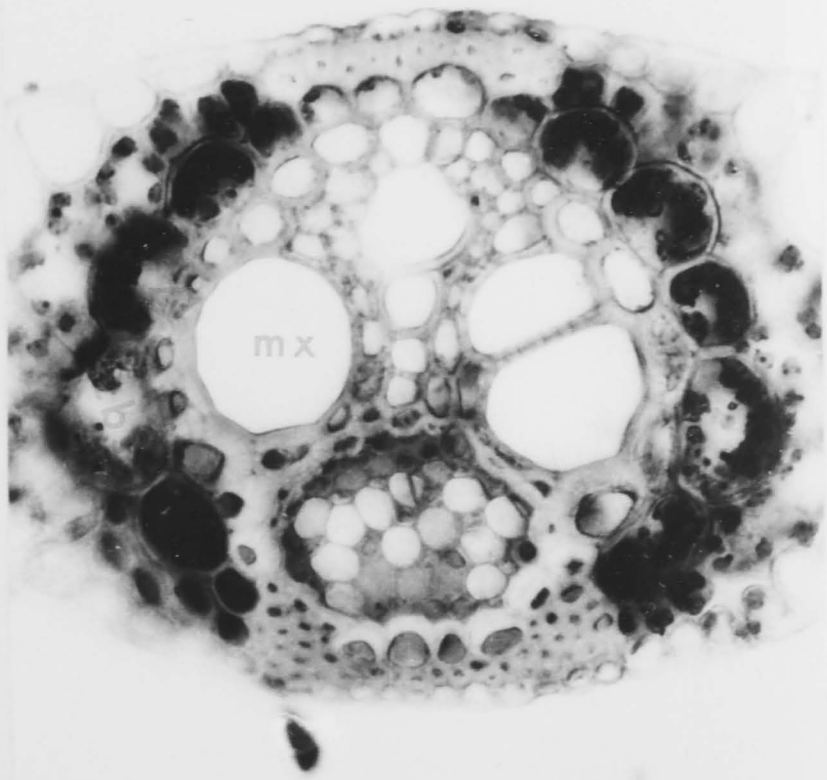
FIG. 4. Transections of grass leaf blades, all C_4 eu-panicoid, showing primary lateral vascular bundles. Adaxial epidermis uppermost. Prepared as in Chapter 3.3.. *A*, *Panicum decompositum*, XyMS+, "NAD-ME type"; herbarium material; note *extra* layer of cells intervening between *mx* and *cbs*; X415. *B*, *Panicum maximum*, XyMS+, "PCK type"; CBS cells larger in transectional area than mesophyll cells; X395. *C*, *Panicum antidotale*, XyMS-, "NADP-ME type"; CBS cells generally smaller in transectional area than mesophyll cells; note *ims* layer; X540. *D*, *Echinochloa crus-galli*, XyMS-, "NADP-ME type"; CBS cells generally smaller in transectional area than mesophyll cells; X395.

Open-head arrows (⇦) indicate cells intervening between the metaxylem vessel element and laterally adjacent chlorenchymatous bundle sheath (CBS) cells (XyMS+). Solid-head arrows (⇩) indicate the metaxylem vessel element in direct contact with laterally adjacent CBS cells (XyMS-). *cbs*, chlorenchymatous bundle sheath; *mx*, metaxylem vessel element; *xl*, extra layer of cells intervening between *mx* and *cbs* (marked with ⇧); *ims*, layer of cells which could be interpreted as an incomplete "mestome sheath" (marked with ⇧).

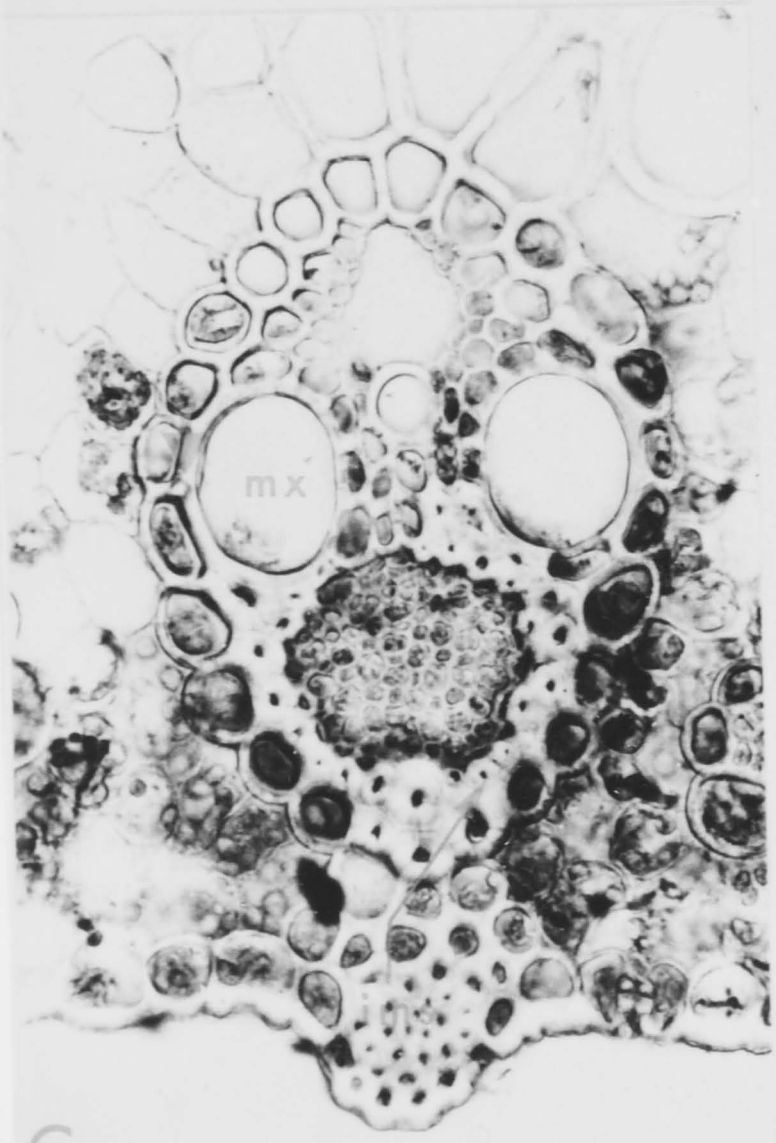
Fig.4



A



B



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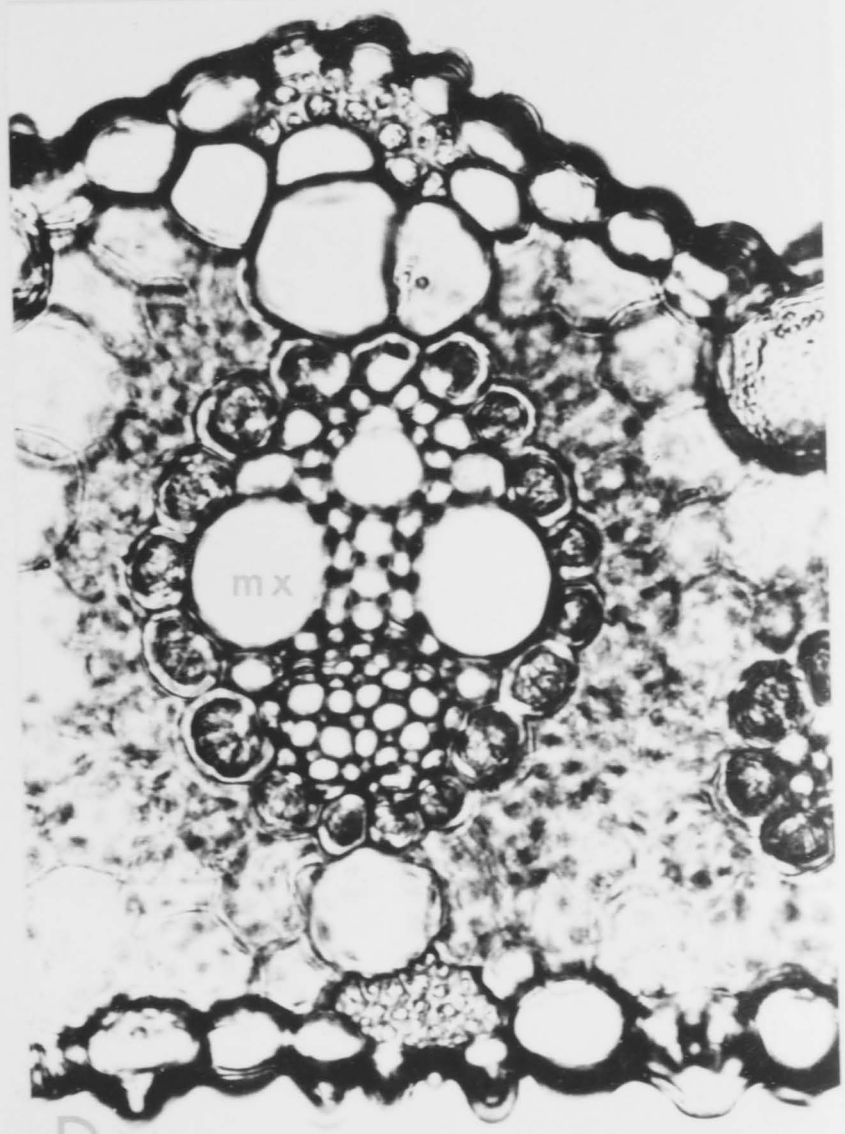
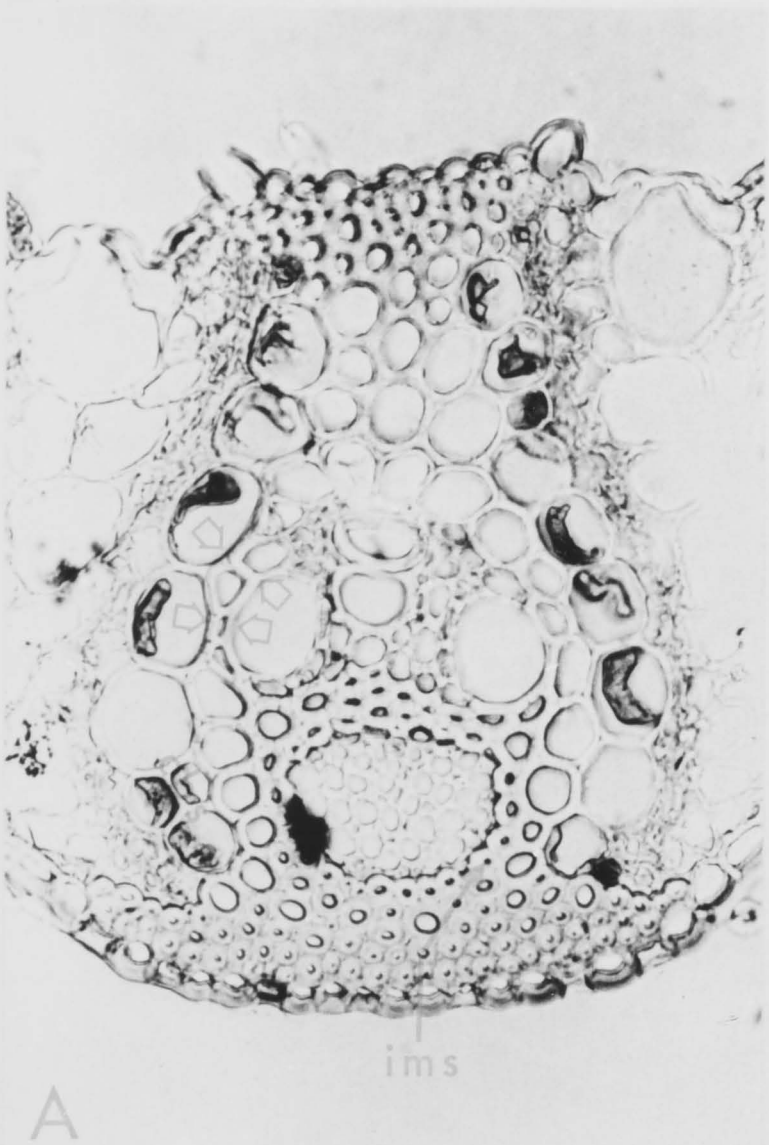


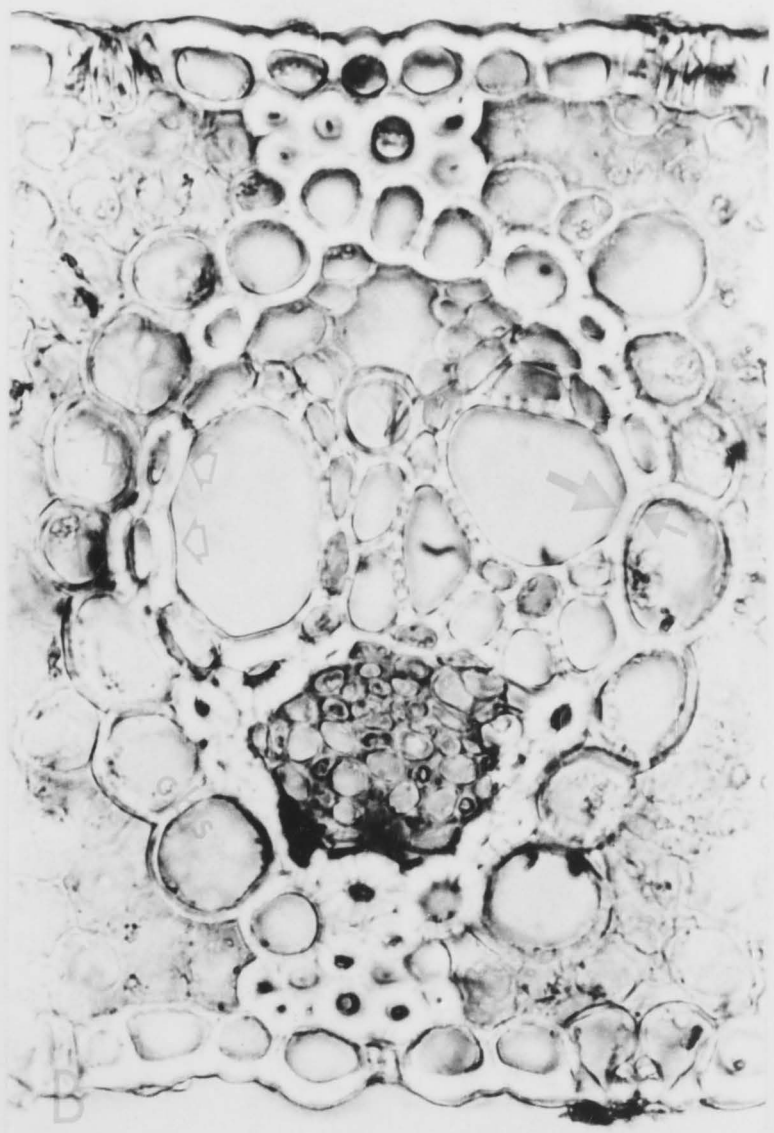
D

FIG. 5. Transections of grass leaf blades, showing primary lateral vascular bundles. Abaxial epidermis at bottom in A, B, and D, and at right in C. Prepared as in Chapter 3.3.. *A, Eragrostis curvula*, C_4, XyMS+, "NAD-ME type", chloridoid (eragrostoid); herbarium material, note *ims* layer; X570. *B, Saccharum officinarum*, C_4, "NADP-ME type", andropogonoid; leaf blades of this species exhibit a variable XyMS condition; the vascular bundle illustrated is XyMS+ on one side and XyMS- on the other; X550. *C, Imperata cylindrica*, C_4, andropogonoid; leaf blades of this species exhibit a variable XyMS condition, the vascular bundle illustrated being XyMS+ on both sides; X475. *D, Amphibromus* sp., C_3, festucoid; XyMS+, as with all other C_3 spp. observed; X575.

Open-head arrows (▽) indicate cells intervening between the metaxylem vessel element and laterally adjacent chlorenchymatous bundle sheath (CBS) cells (XyMS+). Solid-head arrows (↓) indicate a metaxylem vessel element in direct contact with laterally adjacent CBS cells (XyMS-). *ims*, layer of cells which could be interpreted as an incomplete "mestome sheath" (marked with ↑).

Fig.5





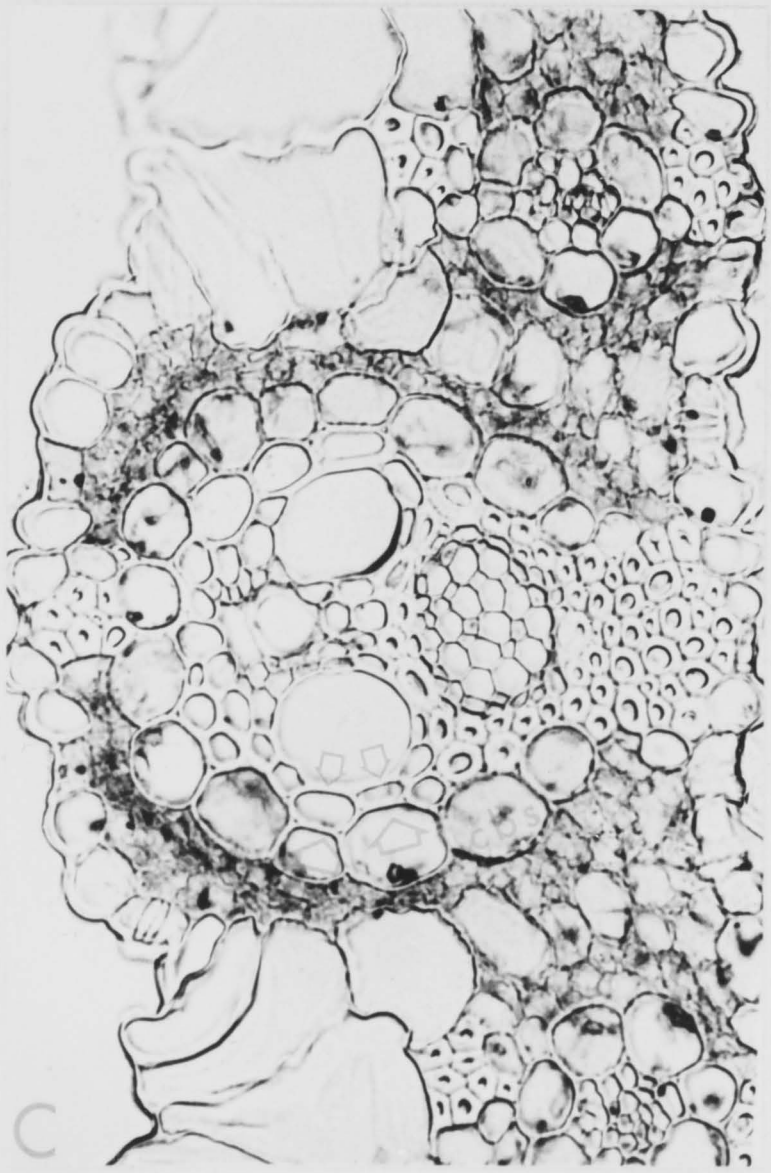
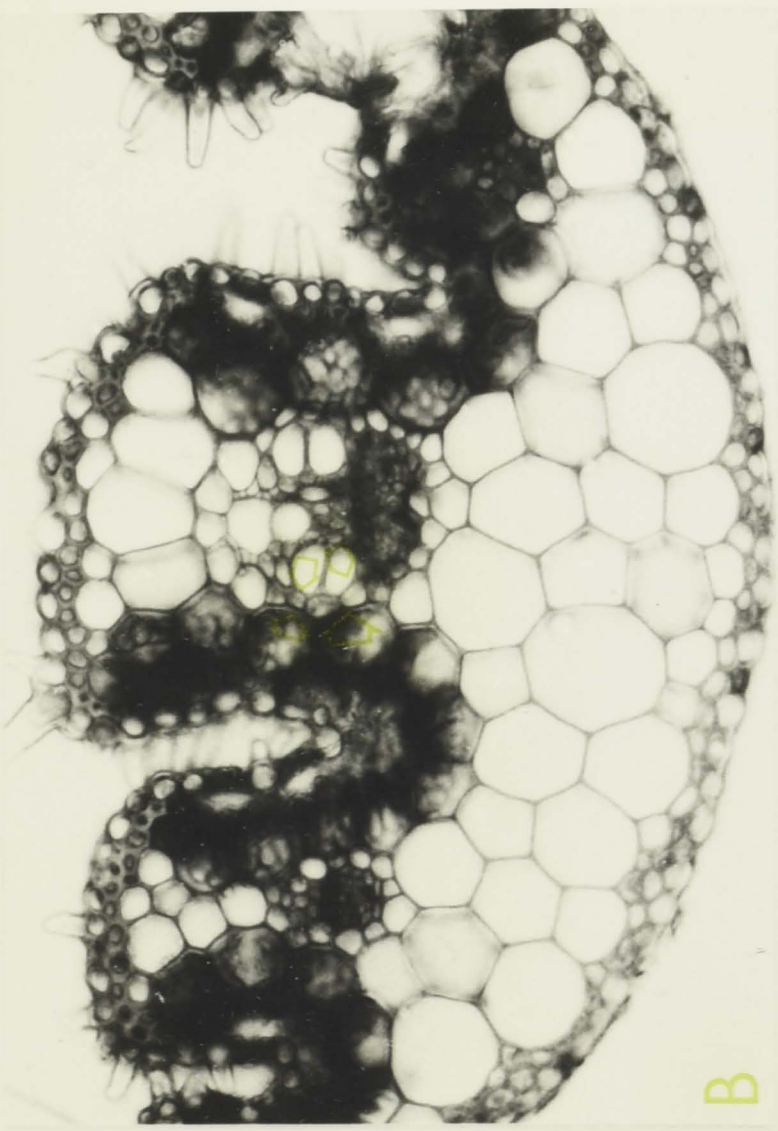


FIG. 6. Transections of C_4 grass leaf blades. Adaxial epidermis uppermost. Prepared as in Chapter 3.3.. *A*, *Eragrostis benthamii*, XyMS+, chloridoid (eragrostoid), predicted "NAD-ME" or "PCK type"; CBS cells larger in transectional area than mesophyll cells; X305. *B*, *Triodia pungens*; XyMS+, danthonioid, predicted "NAD-ME" or "PCK type"; X235. *C*, *Eremochloa bimaculata*, X460, and *D*, *Themeda australis*, X555; both XyMS-, andropogonoid, predicted "NADP-ME type", and having CBS cells smaller in transectional area than mesophyll cells.

For primary lateral vascular bundles, open-head arrows (▽) indicate cells intervening between the metaxylem vessel element and laterally adjacent chlorenchymatous bundle sheath (CBS) cells (XyMS+). Solid-head arrows (▼) indicate a metaxylem vessel element in direct contact with CBS cells (XyMS-).

Fig.6



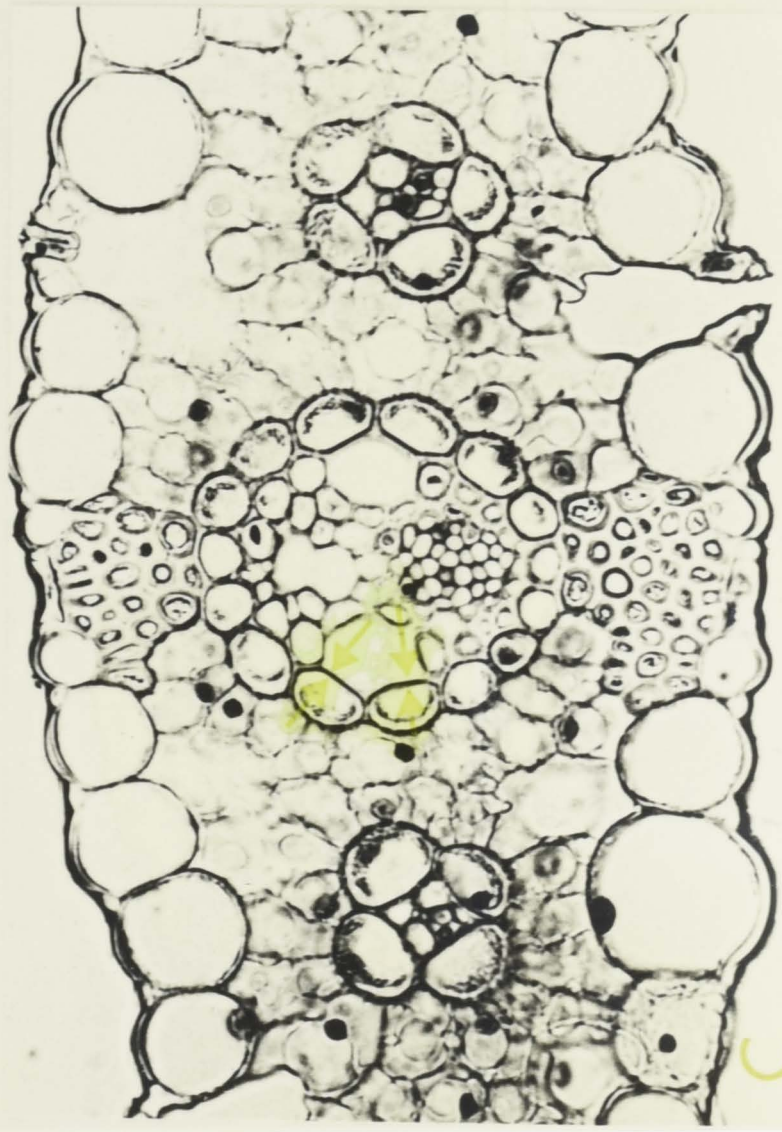


FIG. 7. Hand transections of grass leaf blades, showing immunofluorescent labelling of RuP_2Case in chloroplasts; normal serum control, left; anti-RuP_2Case test, right; adaxial epidermis uppermost. Prepared as in Chapter 5.3.5..
A, *Triticum aestivum* (festucoid), C_3; immunofluorescing chloroplasts in all chlorenchymatous cells in test (control, X445; test, X545). *B*, *Panicum maximum* (eu-panicoid), C_4, XyMS+; immunofluorescing chloroplasts only in PCR cells in test; arrow indicates cells in the XyMS position (both X325). *C*, *Pennisetum villosum* (eu-panicoid), C_4, XyMS-; immunofluorescing chloroplasts only in PCR cells in test; note absence of cells in the XyMS position in extreme right vein (both X250).

pca, PCA cells; *pcr*, PCR cells.

Fig.7

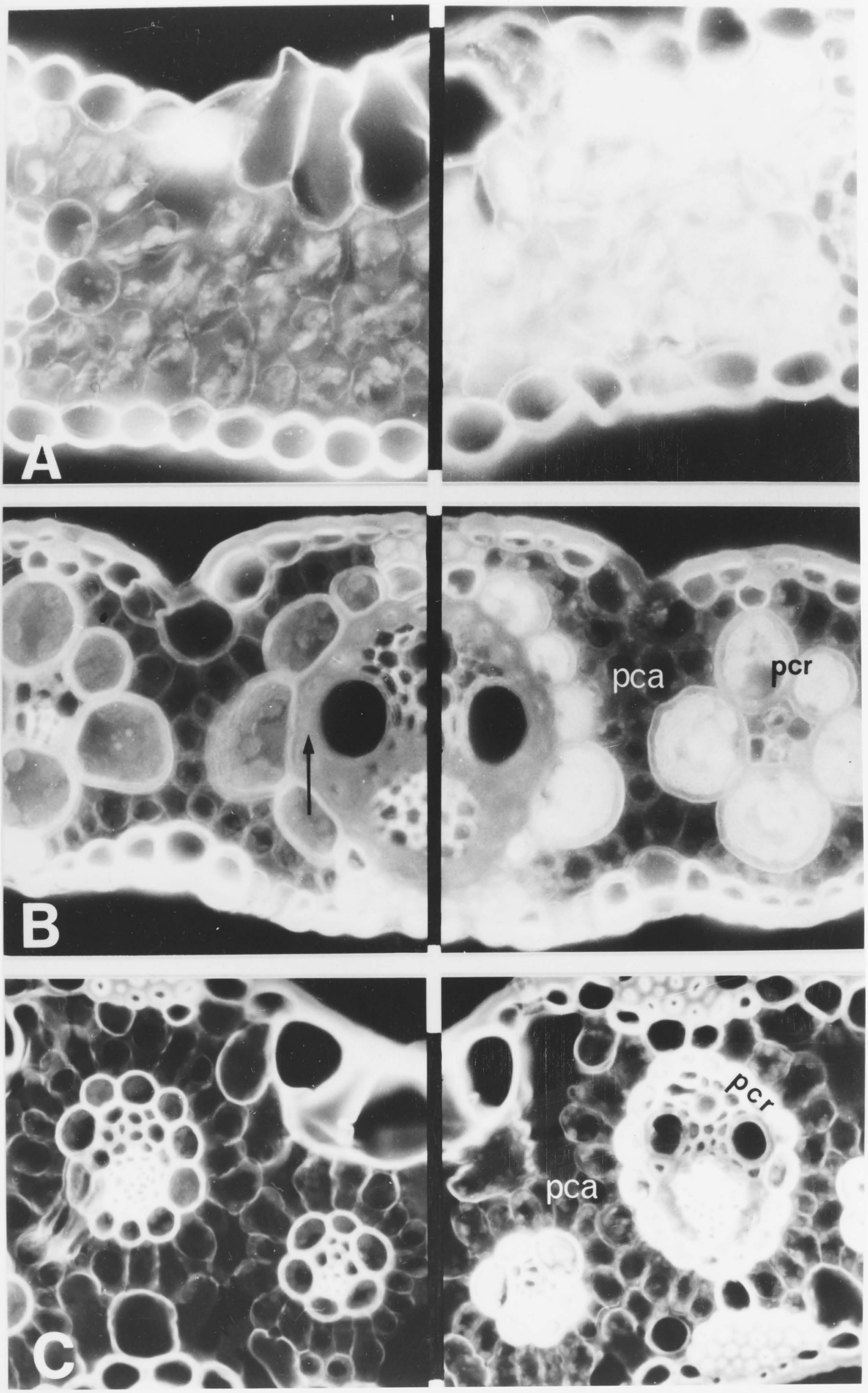


Fig. 8.

FIG. 8. Hand transections of leaf blade of the grass *Arundinella nepalensis* (arundinoid), C_4, XyMS-; showing immunofluorescent labelling of RuP_2Case in chloroplasts; normal serum control, top; anti-RuP_2Case test, bottom; adaxial epidermis uppermost. Prepared as in Chapter 5.3.5.. Arrows indicate isolated PCR cells of longitudinal interveinal strands. Immunofluorescing chloroplasts in PCR sheath *and* strand cells in test. Note weak fluorescence of PCA cell chloroplasts in test cf. control; non-radiate PCA tissue; PCR strand cells not in lateral contact with vascular tissue. The lateral cell count between PCR strand cells laterally is ≤ 2, and the "one cell distant criterion" holds. (Both X520).

Fig.8

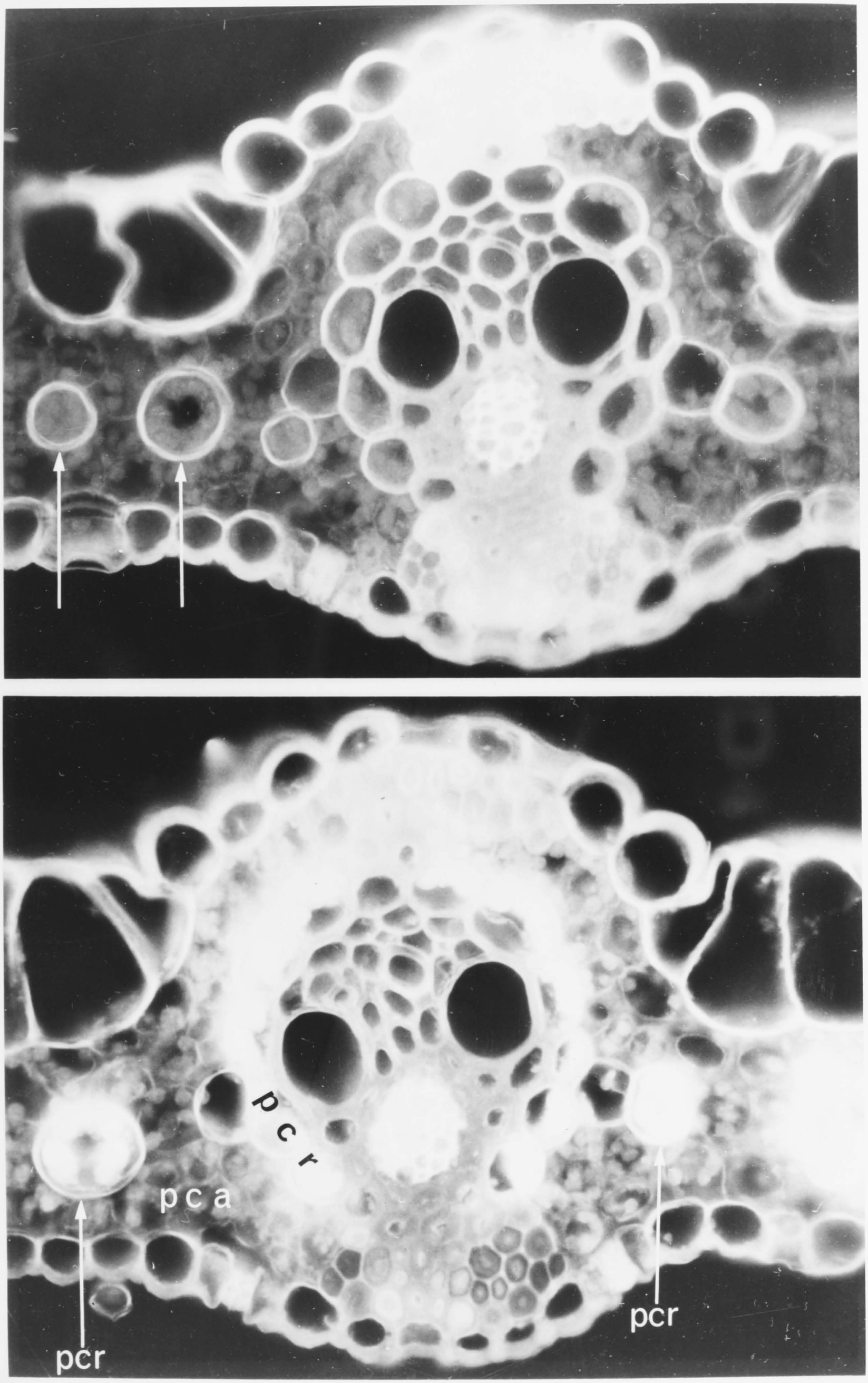


Fig. 9.

FIG. 9. Transections of grass leaf blades; adaxial epidermis uppermost. A and B, fresh tissue stained with I_2 in KI, and observed in brightfield. C, immunofluorescent labelled with anti-RuP_2Case (A/S-1) as in Chapter 5.3.5.. *A, Brachiaria foliosa* (eu-panicoid), C_4; PCR cell chloroplasts only stain for starch; X260. *B, Aristida biglandulosa* (Aristideae), C_4; *all* chlorenchymatous cell chloroplasts stain for starch; X260. *C, Danthonia bipartita* (danthonioid), C_3, XyMS+; the "one cell distant criterion" is violated; the immunofluorescent labelling response is C_3 (i.e., RuP_2Case is present in all chlorenchymatous cell chloroplasts), but the response is only at right, near cut edge (just out of field of view) of transection; X410.

Fig.9

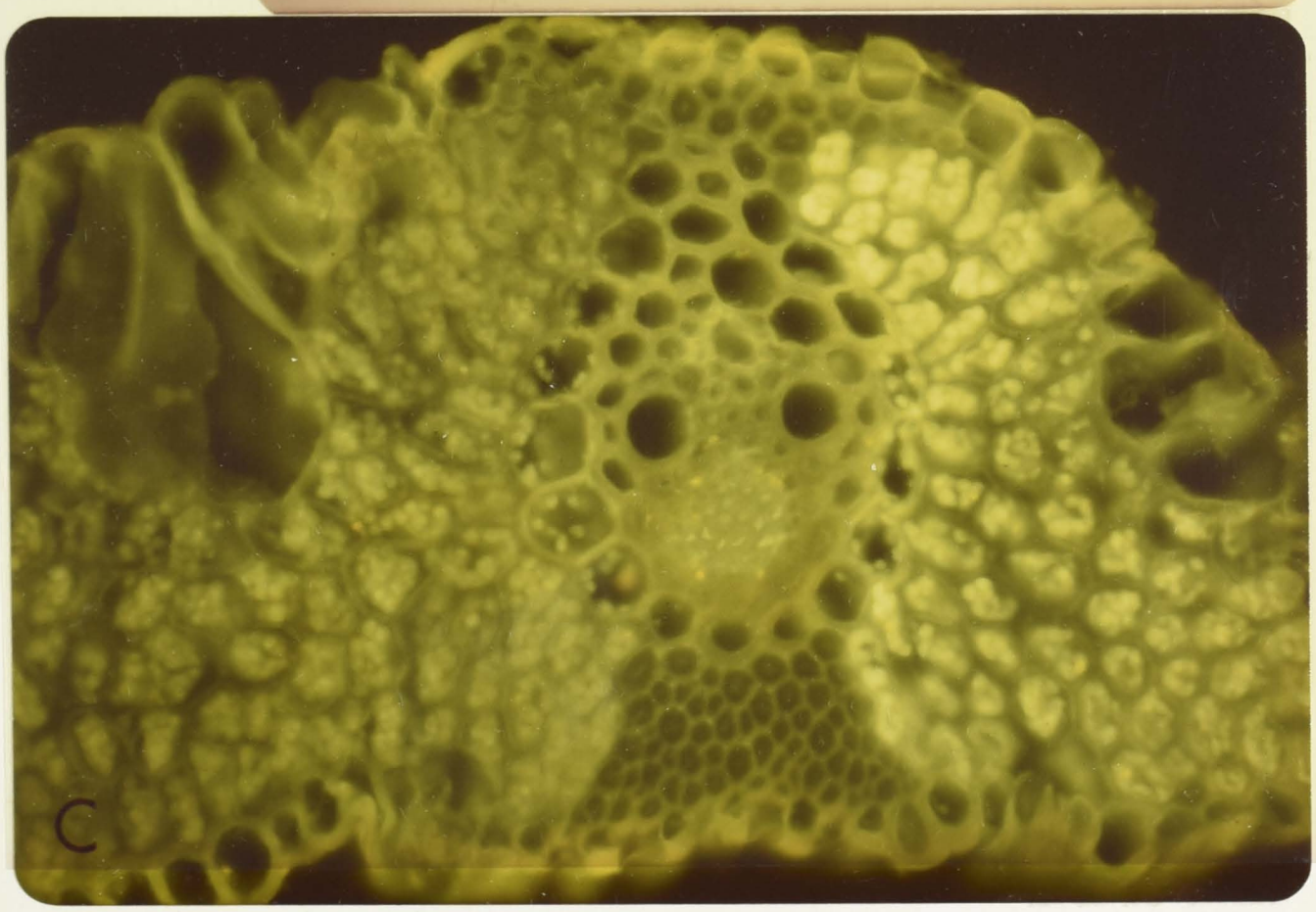


Fig. 10.

FIG. 10. Immunofluorescent labelling of ribulose-1,5-bisphosphate carboxylase in hand transections of leaf blades of *Digitaria brownii* (Gramineae, eu-panicoid, XyMS-), exhibiting "classical" C_4 leaf anatomy; abaxial epidermis at bottom; prepared as in Chapter 5.3.5.; all X650.

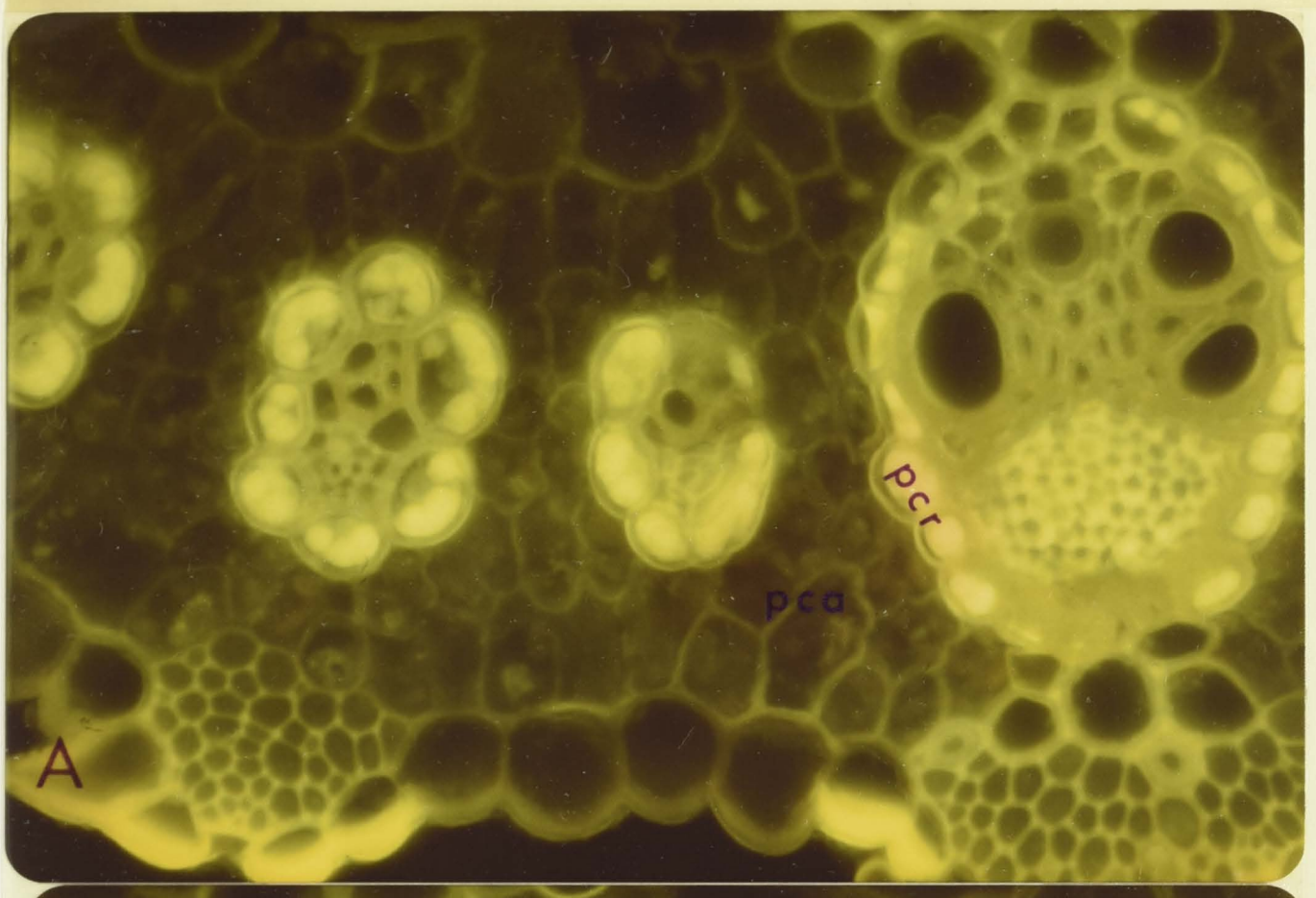
A, anti-wheat RuP_2Case serum test (A/S-5), showing immunofluorescence in "bundle sheath" (PCR) cell chloroplasts only; note the similarity of PCR cell chloroplast immunofluorescence and cuticle autofluorescence, the latter being exhibited in both controls.

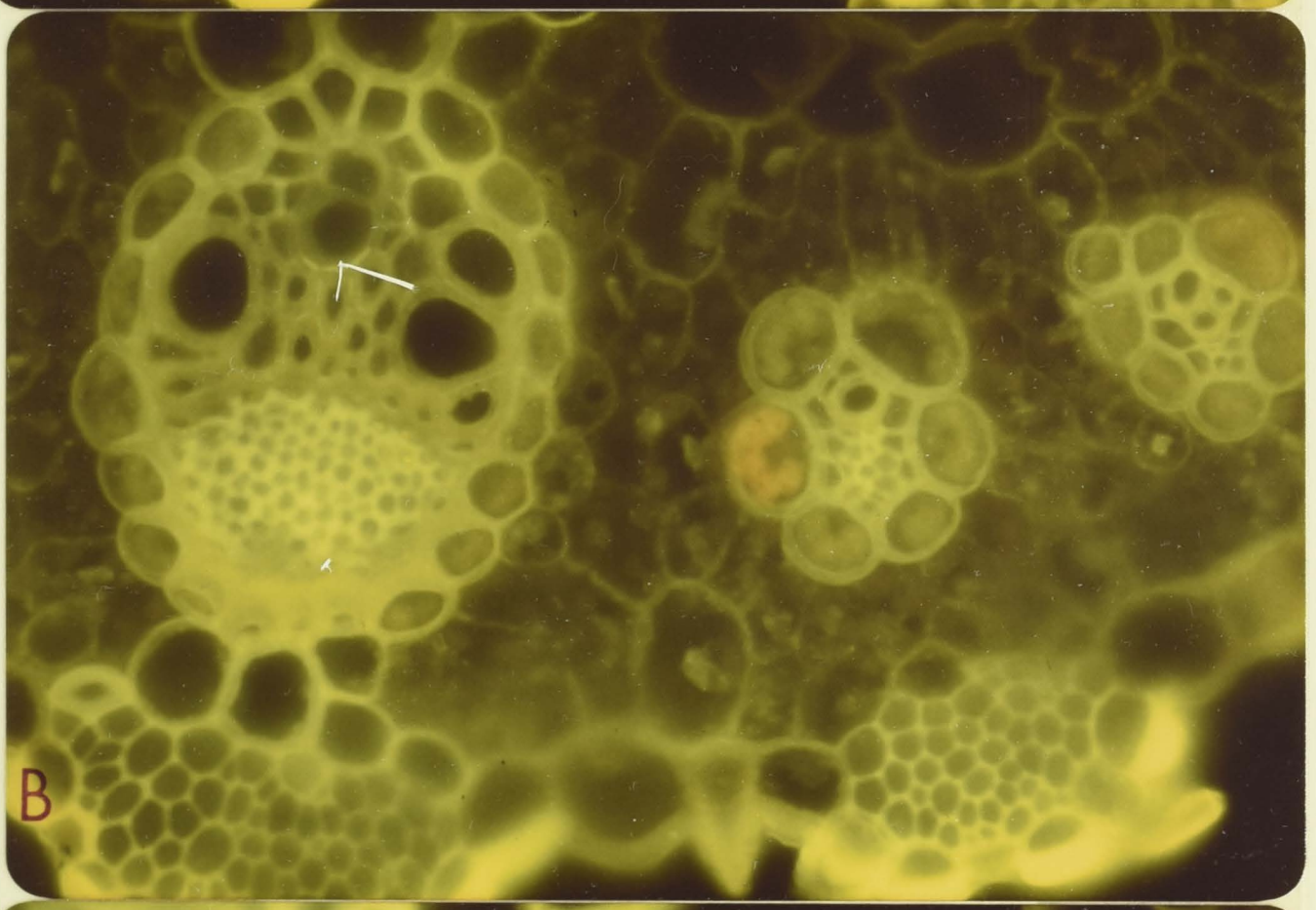
B, normal serum control, showing lack of immunofluorescence in all cell chloroplasts.

C, autofluorescence control.

In all transections, the "one cell distant criterion" holds; PCR cells are generally smaller in transectional area than PCA cells.

Fig.10





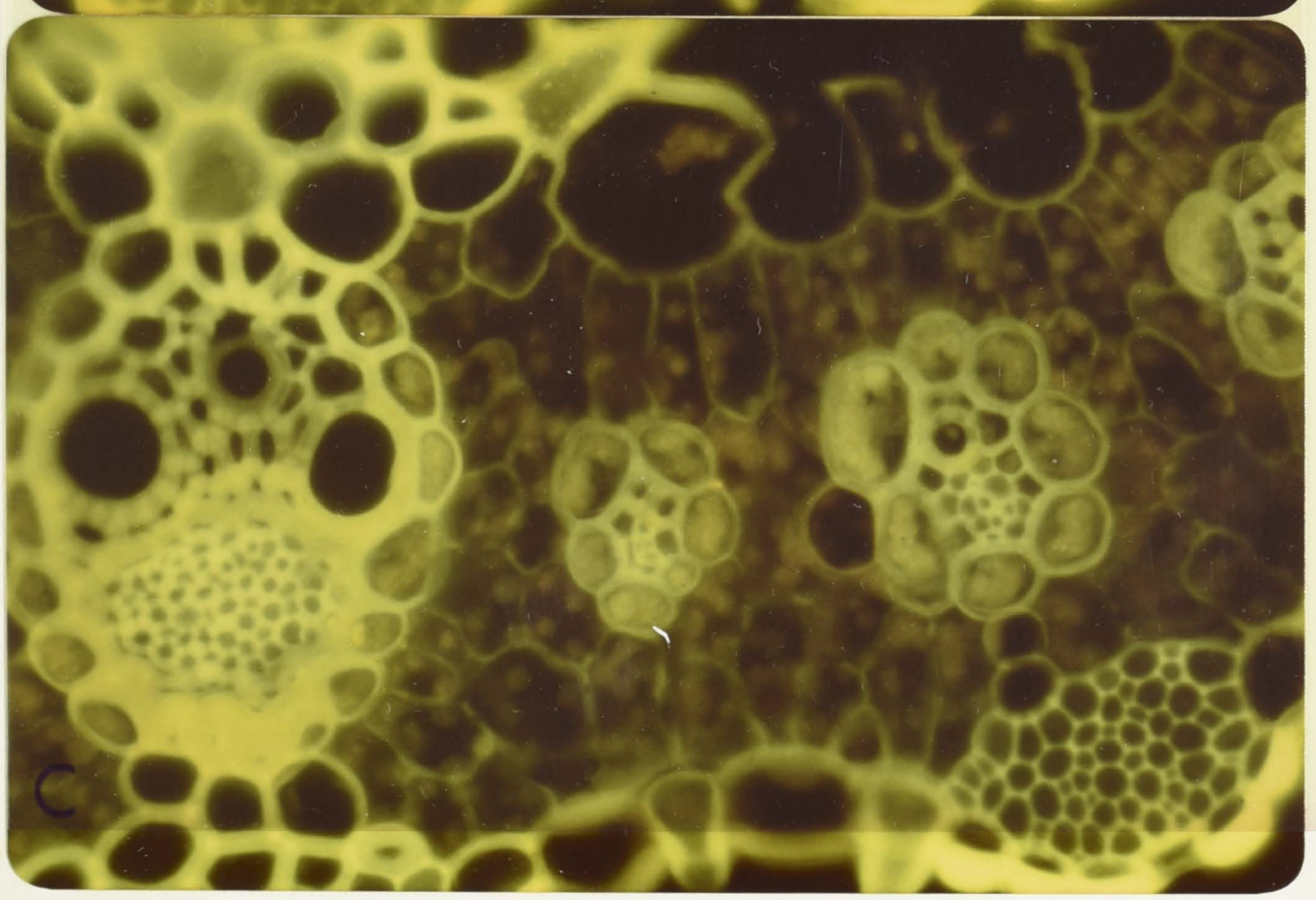


Fig. 11.

FIG. 11. Immunofluorescent labelling of ribulose-1,5-bisphosphate carboxylase in hand transections of leaf blades of *Sporobolus fimbriatus* (Gramineae, chloridoid, XyMS+), exhibiting "classical" C_4 leaf anatomy; abaxial epidermis at bottom; prepared as in Chapter 5.3.5..

A, anti-wheat RuP_2Case serum test (A/S-3), showing immunofluorescence in "bundle sheath" (PCR) cell chloroplasts only; X255.

B, normal serum control; X410.

C, autofluorescence control; X255.

In all transections, the "one cell distant criterion" holds; PCR cells are generally larger in transectional area than PCA cells.

Fig.11

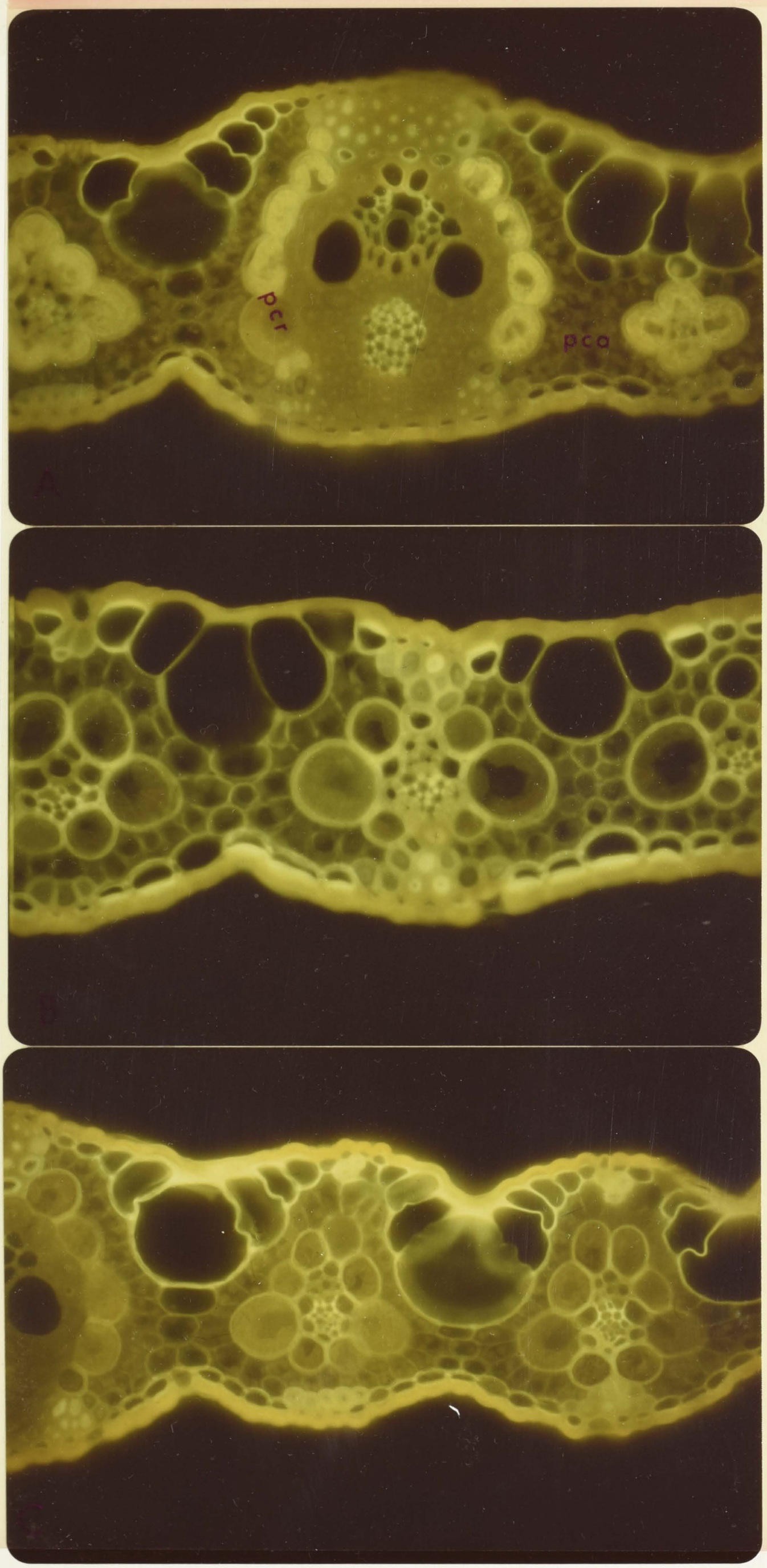
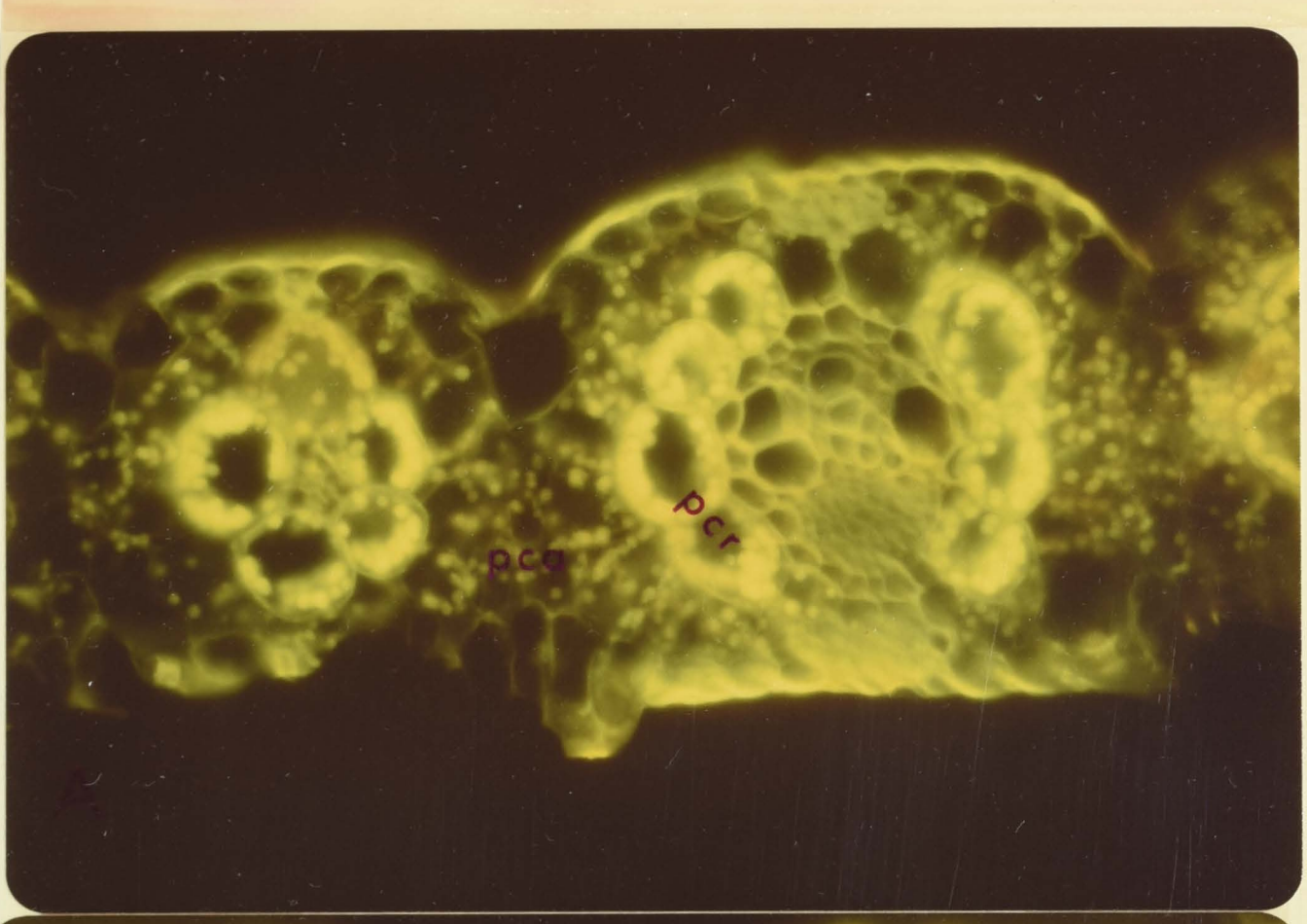
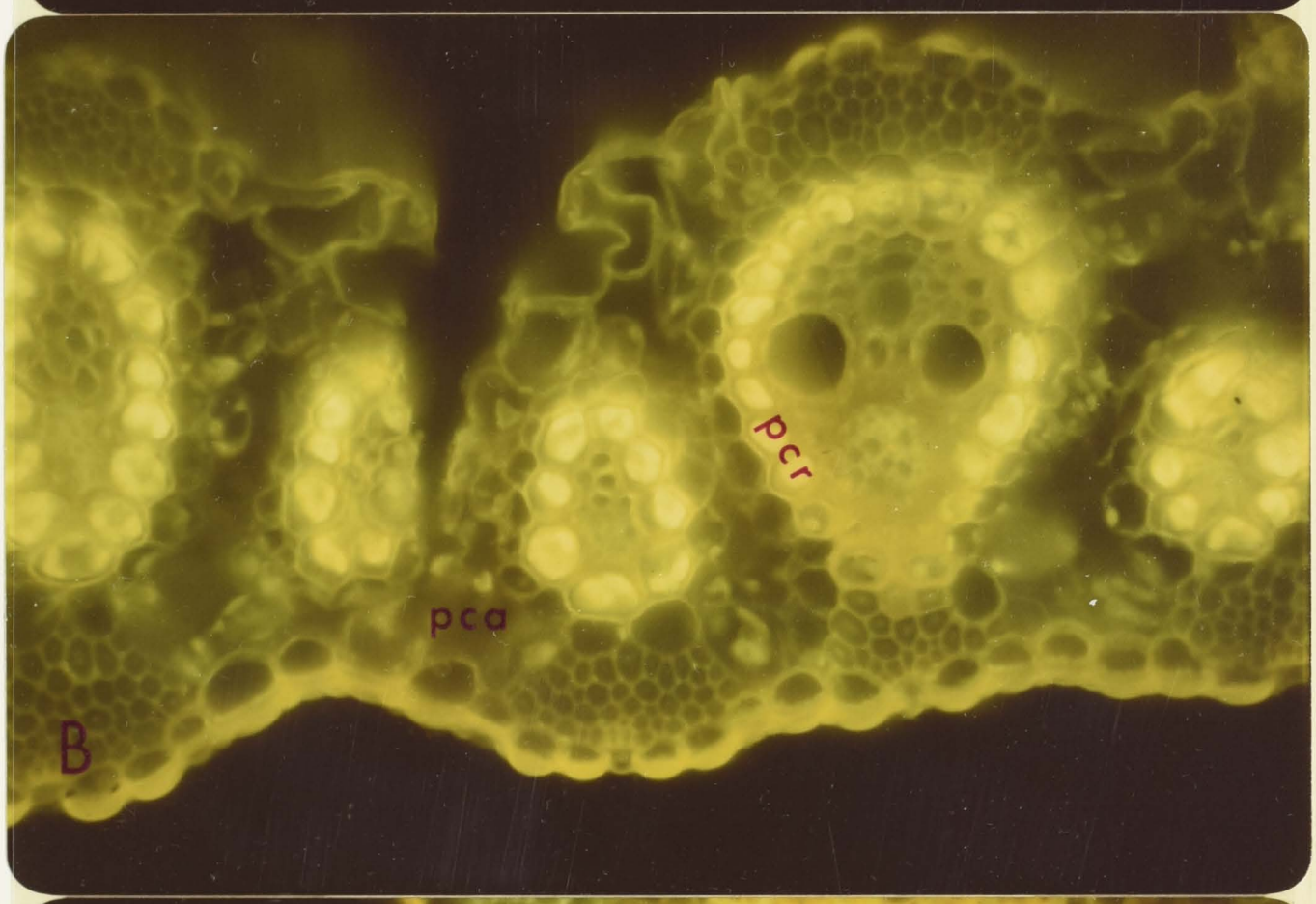


Fig. 12.

FIG. 12. Immunofluorescent labelling of RuP_2Case in hand transections of leaf blades of C_4 eu-panicoid grass species; abaxial epidermis at bottom; prepared as in Chapter 5.3.5..
A, *Brachiaria foliosa*, showing a clear C_4 labelling response even when anti-RuP_2Case serum has been diluted 50X (A/S-3); slight PCA cell chloroplast immunofluorescence is weak relative to that of PCR; X255. *B*, *Alloteropsis semialata*, anti-RuP_2Case test (A/S-5), showing a clear C_4 labelling response when post-section fixed; this species exhibits "non-classical" C_4 leaf anatomy, the PCR sheath consisting the inner of the two recognisable bundle sheaths (cf. control below for clearer micrograph of structure); X255.
C. Alloteropsis semialata, normal serum control; "non-classical" C_4 leaf anatomy; PCR sheath separated from PCA cells by layer of almost chloroplast-free cells which are smaller in transectional area than PCR cells; X410.

Fig.12





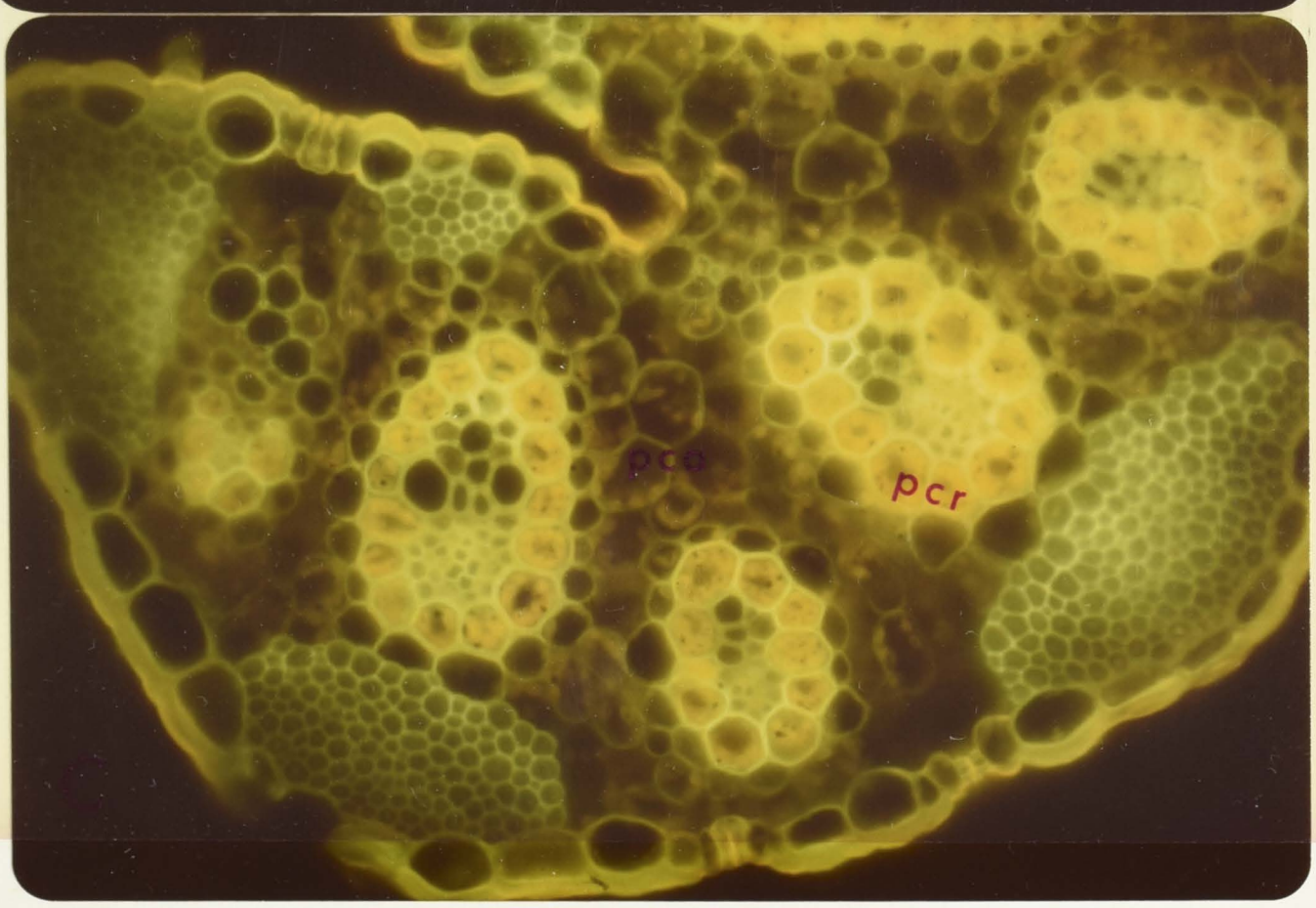


Fig. 13.

FIG. 13. Immunofluorescent labelling of RuP_2Case in hand transections of leaf blades of *Aristida ramosa* (Gramineae, Aristideae, XyMS-), exhibiting "non-classical" C_4 leaf anatomy; adaxial epidermis uppermost; prepared as in Chapter 5.3.5.; both X410.

A, anti-wheat RuP_2Case test (A/S-4), showing a clear labelling response when post-section fixed; PCR cells constitute *both* recognisable bundle sheaths.

B, normal serum control.

Fig.13

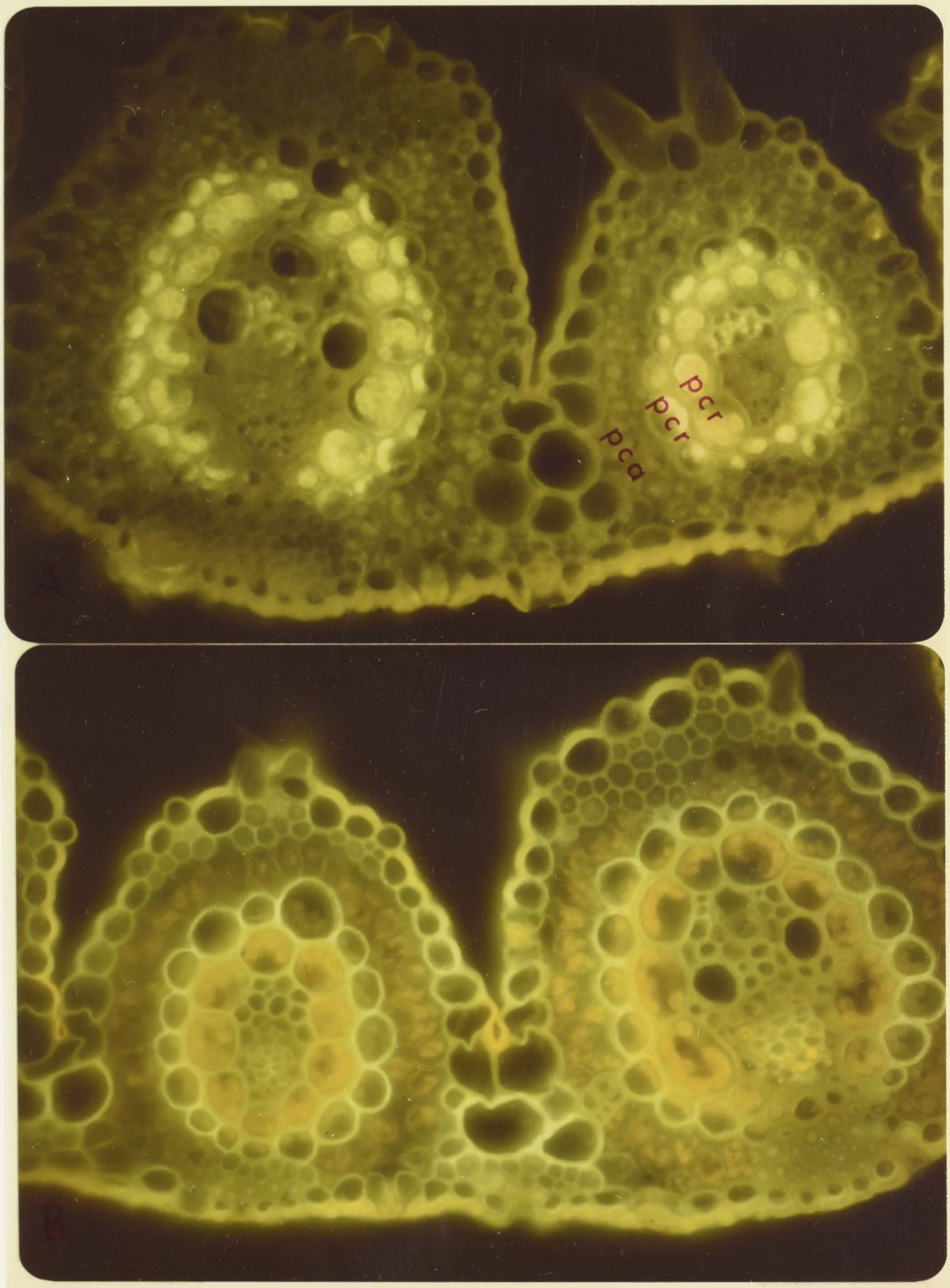
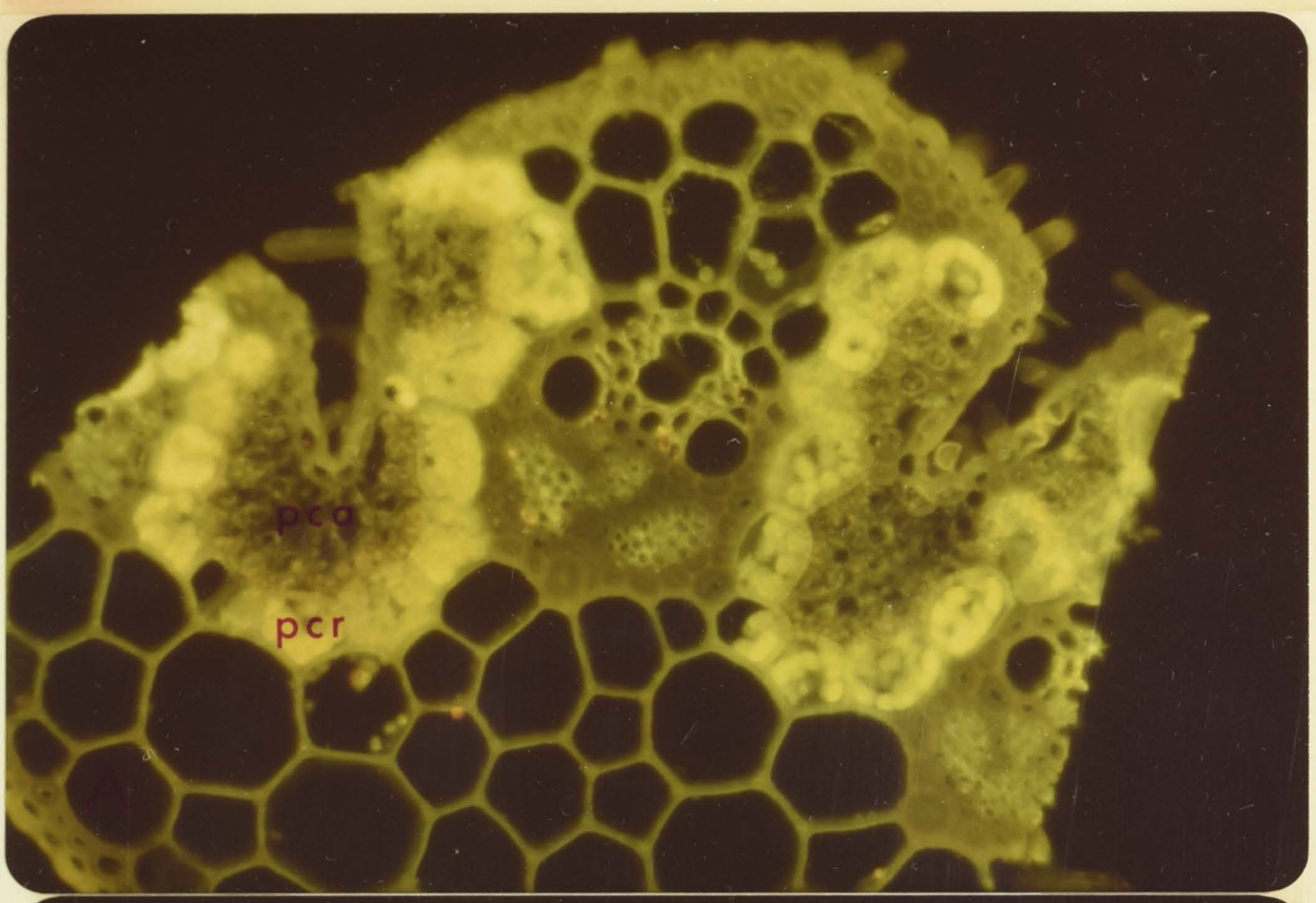


Fig. 14.

FIG. 14. Immunofluorescent labelling of RuP_2Case in hand transections of leaf blades of C_4 species with "non-classical" C_4 leaf anatomy; adaxial epidermis uppermost in *A* and *B*; leaf of *C* is cylindraceous; prepared as in Chapter 5.3.5.. *A*, *Triodia pungens* (Gramineae, danthonioid), anti-RuP_2Case test (A/S-1); showing a clear response when adaxial epidermis has been nicked prior to fixation; PCR cells constitute a bundle sheath and its extensions, which "drape" between adjacent vascular bundles; not all PCR cells are juxtaposed to vascular tissue; X255. *B*, *Triodia pungens* (Gramineae, danthonioid, XyMS+, probably NAD-ME type or PCK type), normal serum control; PCR cells constitute a bundle sheath with its extensions, which sometimes "drape" between adjacent vascular bundles; not all PCR cells are juxtaposed to vascular tissue; X160. *C*, *Salsola kali* (Chenopodiaceae), anti-spinach large subunit RuP_2Case test (A/S-7); immunofluorescence largely restricted to cell chloroplasts of innermost of the two peripheral chlorenchymatous cell layers which surround the entire cylindraceous leaf; PCR cells not always associated with vascular tissue; PCA cell chloroplasts immunofluoresce slightly, but weak relative to PCR; X1015.

Fig.14



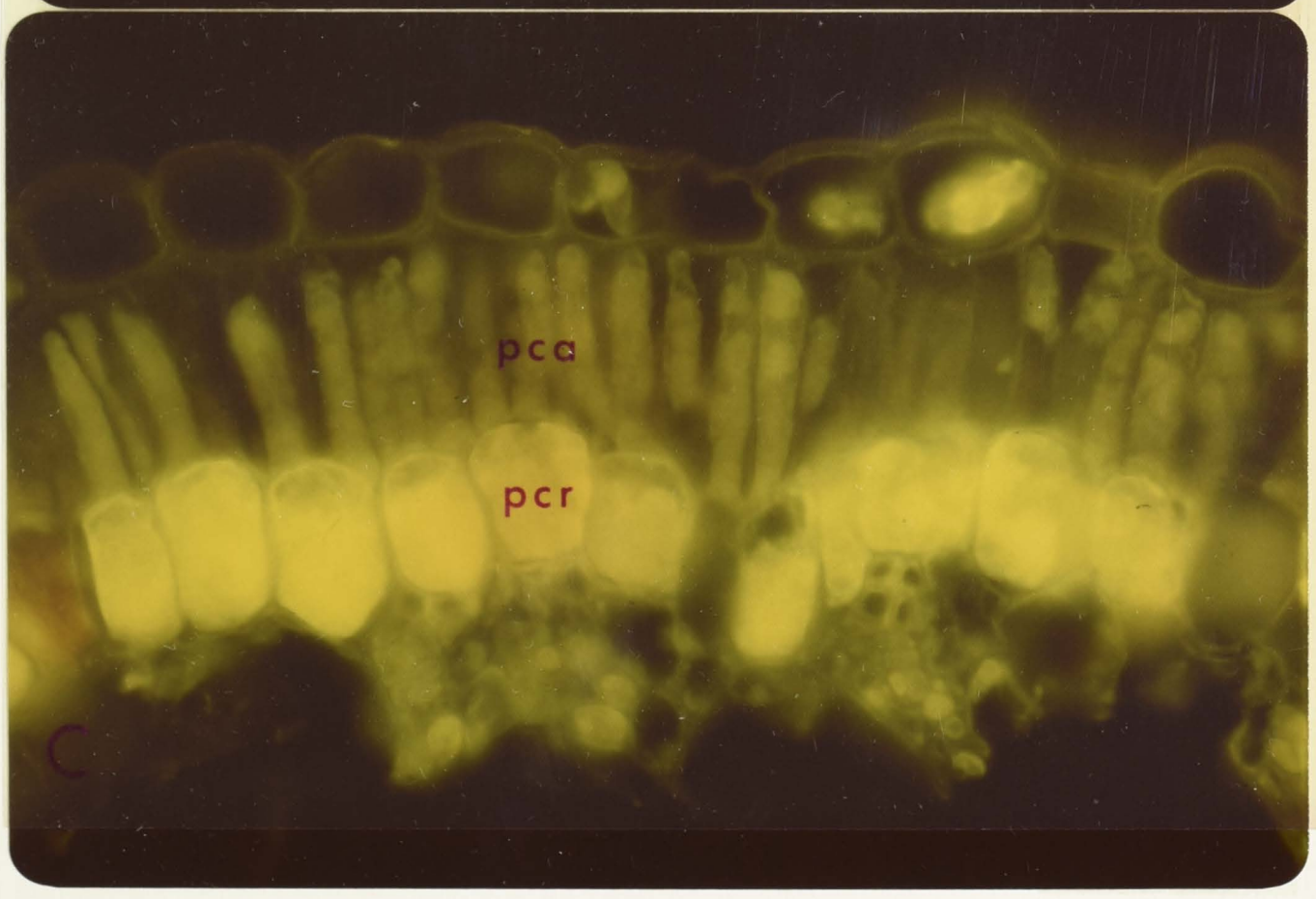


Fig. 15.

FIG. 15. Immunofluorescent labelling of RuP_2Case in hand transections of leaf blades of C_4 Cyperaceae with "non-classical" C_4 leaf anatomy; abaxial epidermis at bottom; prepared as in Chapter 5.3.5.. *A, Cyperus sanguinolentus,* anti-spinach large subunit RuP_2Case (A/S-7); immunofluorescence restricted to innermost of the two recognisable bundle sheaths; PCR tissue separated from PCA tissue, therefore, by the single layer of relatively small, apparently chloroplast-free cells (distance <10 μm); PCR sheath interrupted laterally by metaxylem vessels in large vascular bundle; X650.
B, Fimbristylis dichotoma, anti-spinach native RuP_2Case (A/S-6); immunofluorescence restricted to innermost of the three recognisable (clear in vein at left) sheaths; PCR tissue is separated from PCA tissue by a layer of relatively small, apparently chloroplast-free cells; PCR sheath interrupted laterally by metaxylem vessels in large vascular bundles; X255.

Fig.15

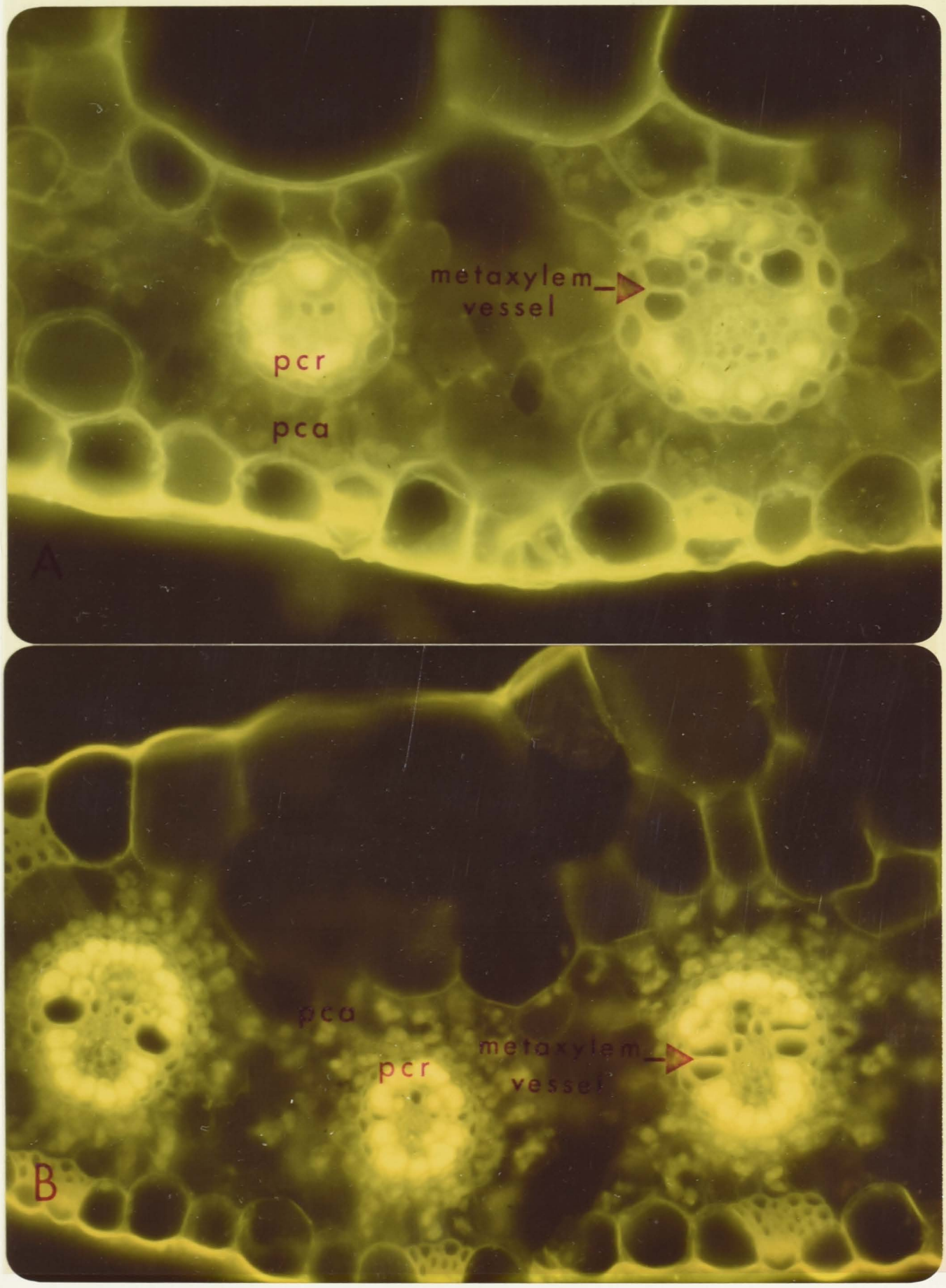
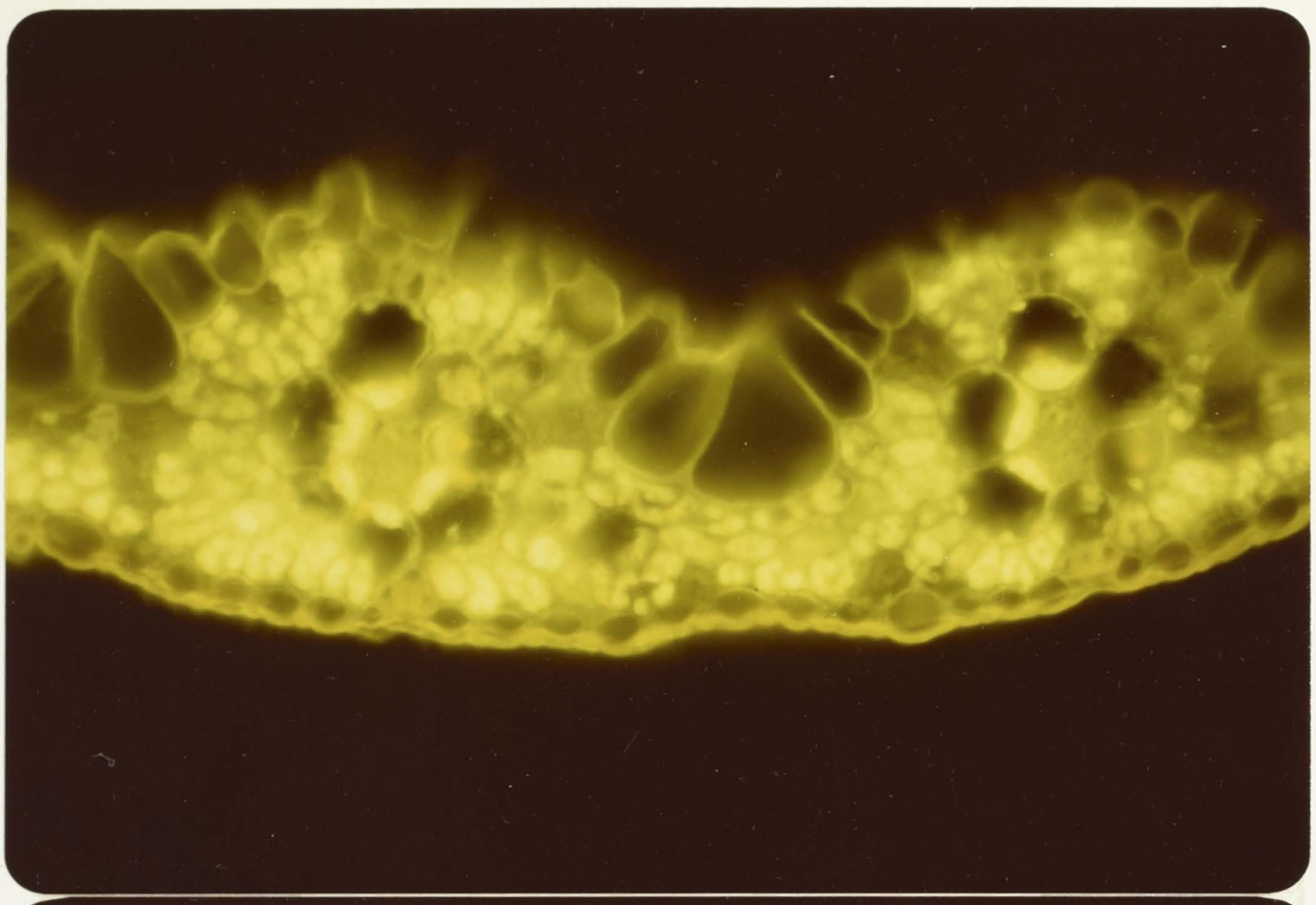


Fig. 16.

FIG. 16. Immunofluorescent labelling of RuP_2Case in hand transections of leaf blades of a reputed C_3/C_4 intermediate, *Panicum milioides* (Gramineae, eu-panicoid, XyMS+); adaxial epidermis uppermost; prepared as in Chapter 5.3.5.; all X410. The lateral cell count is >4. *A*, anti-wheat RuP_2Case test (A/S-1), showing that chloroplasts of all chlorenchymatous cells immunofluoresce, as in C_3 species. *B*, normal serum control. *C*, autofluorescence control.

Fig.16

A



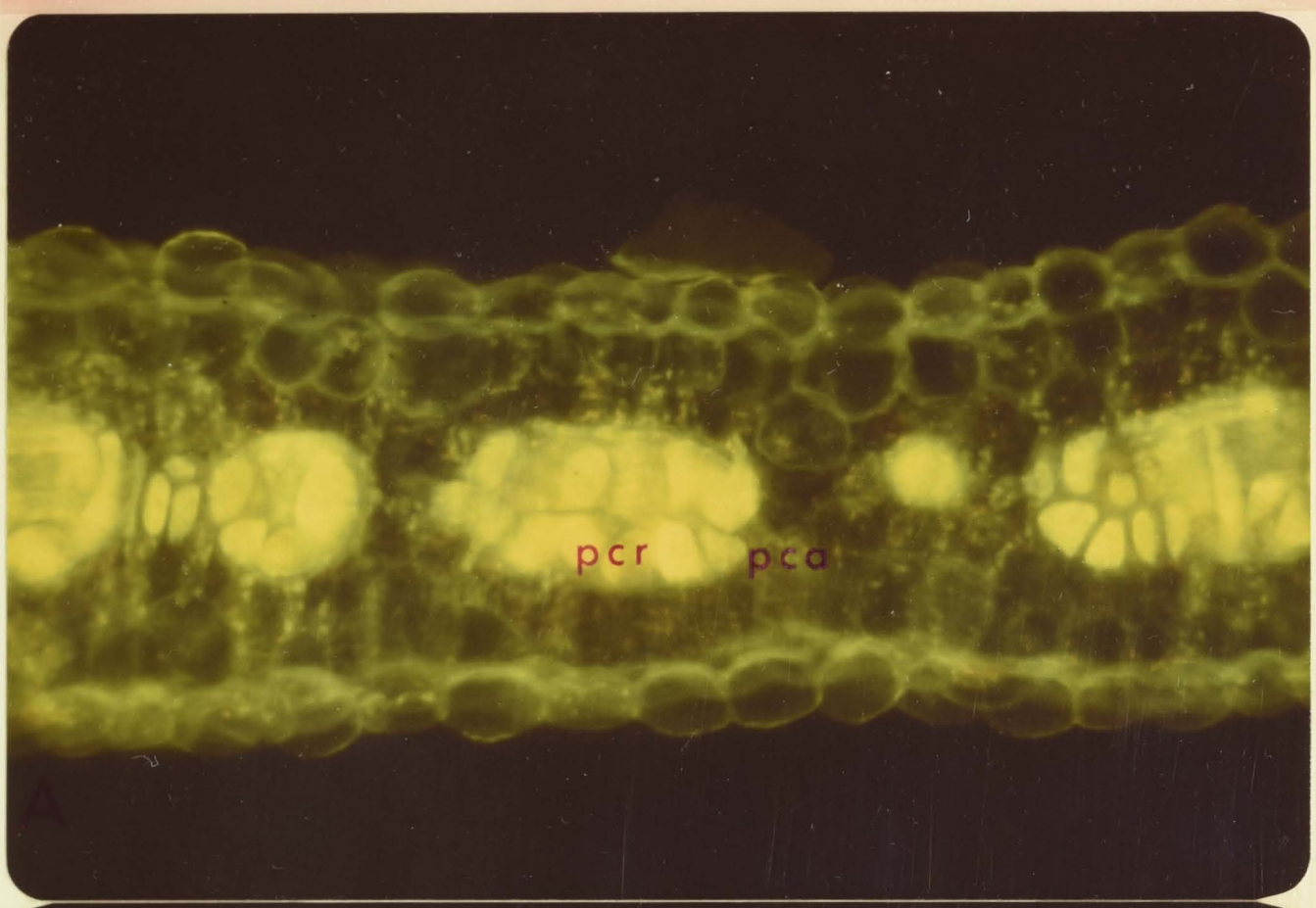
B

C

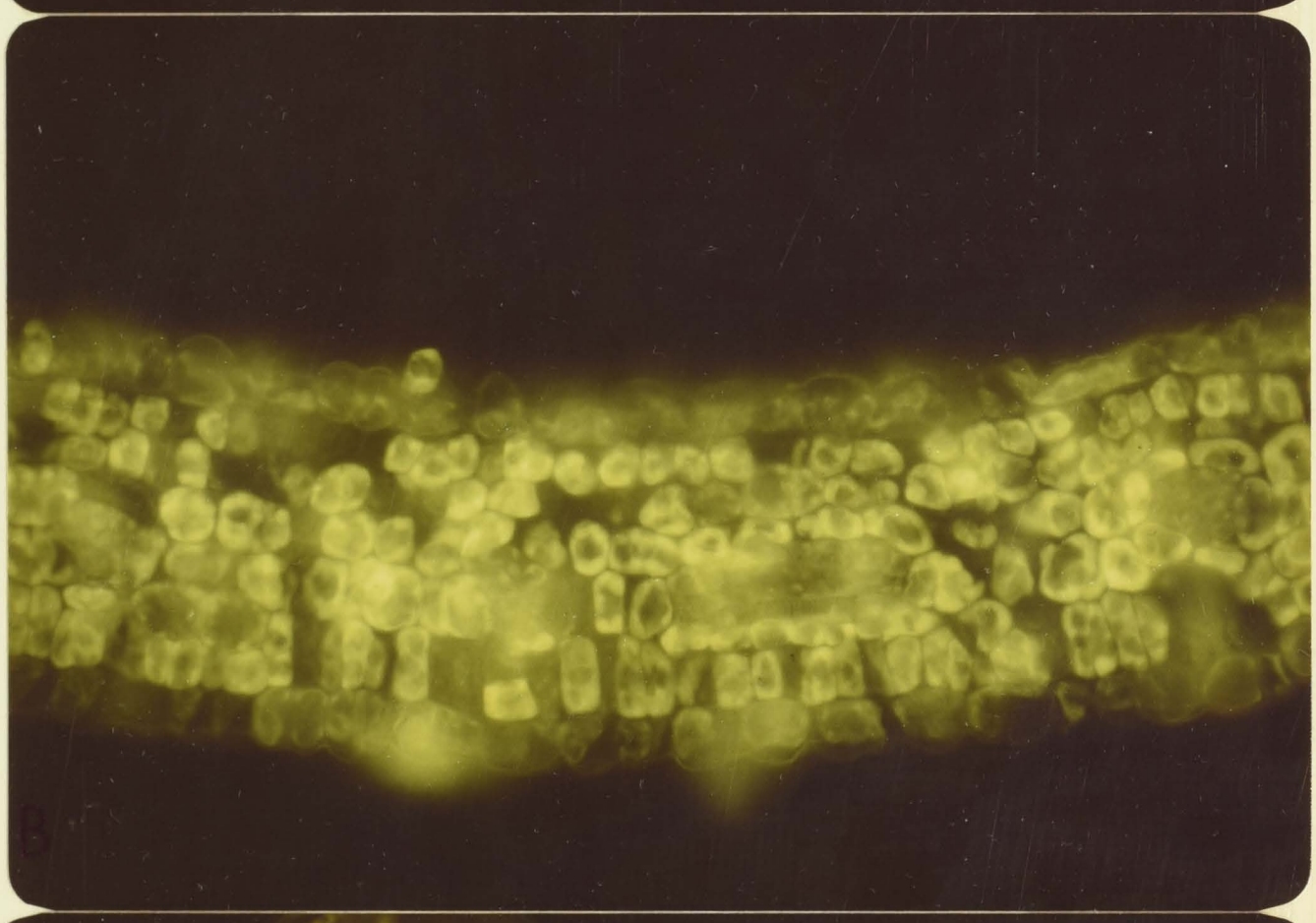
Fig. 17.

FIG. 17. Immunofluorescent labelling of RuP_2Case in hand transections of leaf blades of *Atriplex* spp. and an F_1 hybrid (Chenopodiaceae); prepared as in Chapter 5.3.5.. *A*, *Atriplex rosea*, C_4, anti-wheat RuP_2Case test (A/S-1); immunofluorescence restricted to "bundle sheath" (PCR) cell chloroplasts; X255. *B*, *Atriplex hastata*, C_3, anti-wheat RuP_2Case test (A/S-3); chloroplasts of all chlorenchymatous cells immunofluoresce; X255. *C*, *Atriplex* F_1 hybrid (*A. rosea* x *A. patula* spp. *hastata*), anti-wheat RuP_2Case test (A/S-4); chloroplasts of all chlorenchymatous cells immunofluoresce; bundle sheath cells contain numerous chloroplasts; X410.

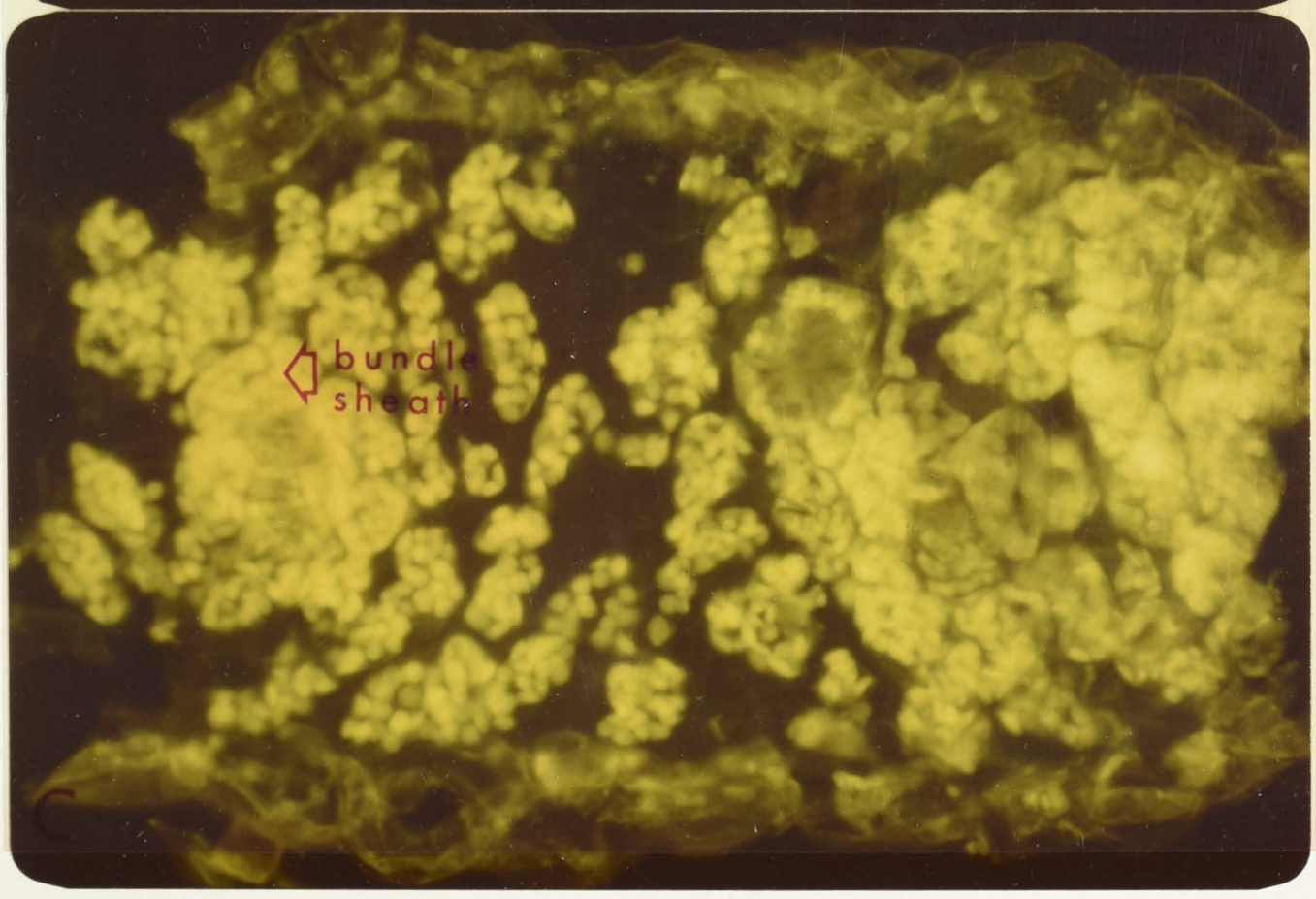
Fig.17



A



B



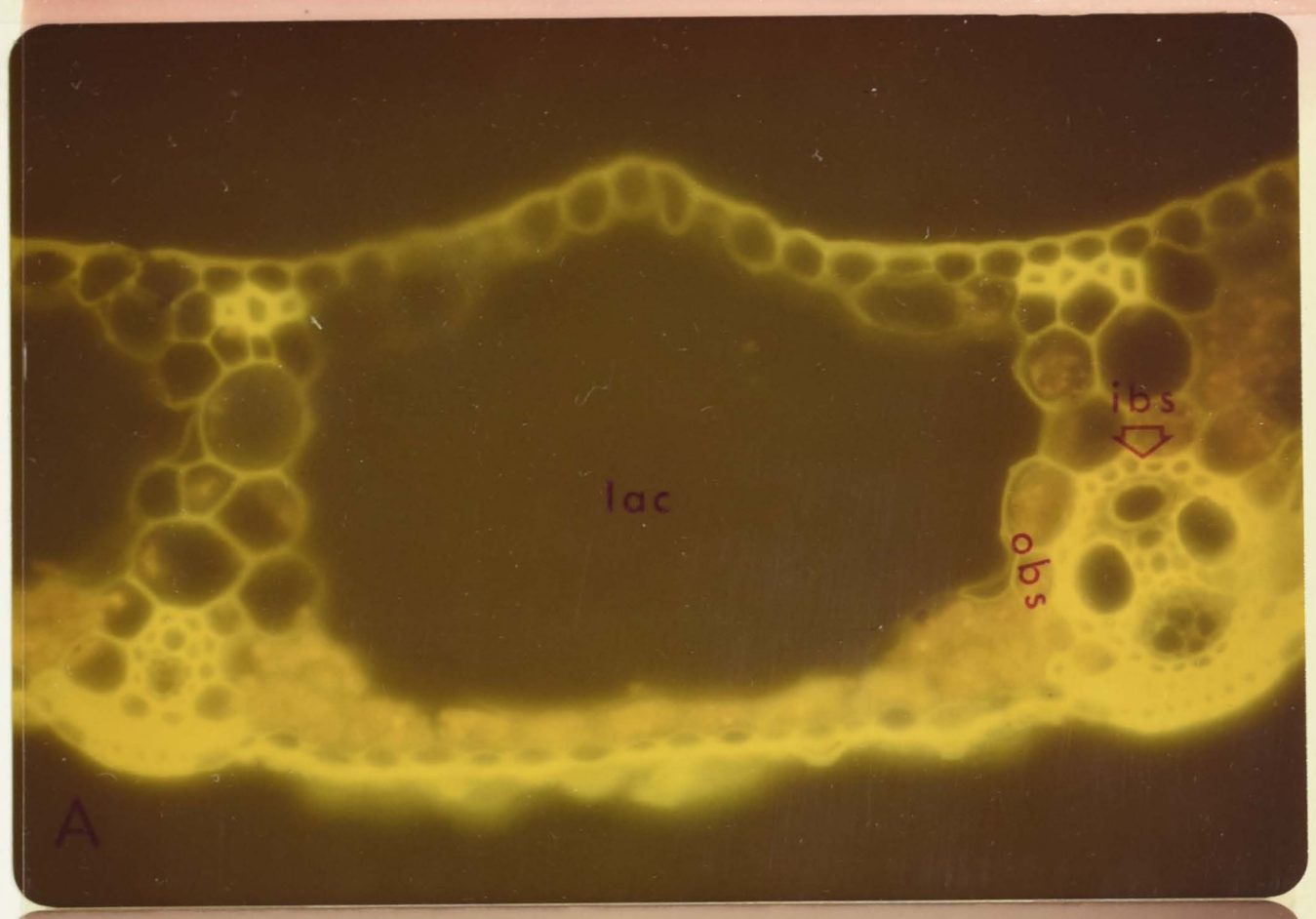
C

Fig. 18.

FIG. 18. Transections of grass leaf sheaths; adaxial epidermis uppermost. *A*, *Oryza sativa* (oryzoid, C_3); chlorenchyma and vascular bundles abaxially situated; chlorenchyma continuous between vascular bundles laterally, the lateral cell count >4; note large lacuna; sheath of cells in inner bundle sheath position (arrowed) retains integrity adaxially; stained in phenolic Bismarck Brown and observed with epifluorescence as in Chapter 5.3.5. for autofluorescence controls; X325. *B*, *Amphibromus neesii* (festucoid, C_3; cf. blade Fig. 3C); chlorenchyma and vascular bundles abaxially situated; chlorenchyma continuous between vascular bundles laterally, the lateral cell count >4; sclerenchyma abaxial to vascular tissue; note lacuna with bordering torn cell wall, and stoma in abaxial epidermis; sheath of cells in inner bundle sheath position (arrowed) retains identity adaxially cf. cells in outer bundle sheath position (arrowed); fresh material, observed in brightfield; X415. *C*, *Brachiaria foliosa* (eu-panicoid, C_4; cf. blade Fig. 9A); large cells between vascular bundles contain very few chloroplasts; bundle sheath cell chloroplasts stained for starch; stained with I_2 in KI; brightfield, X265.

lac, lacuna; *st*, stoma; *obs*, outer bundle sheath; *ibs*, inner bundle sheath; *s*, sclerenchyma.

Fig. 18



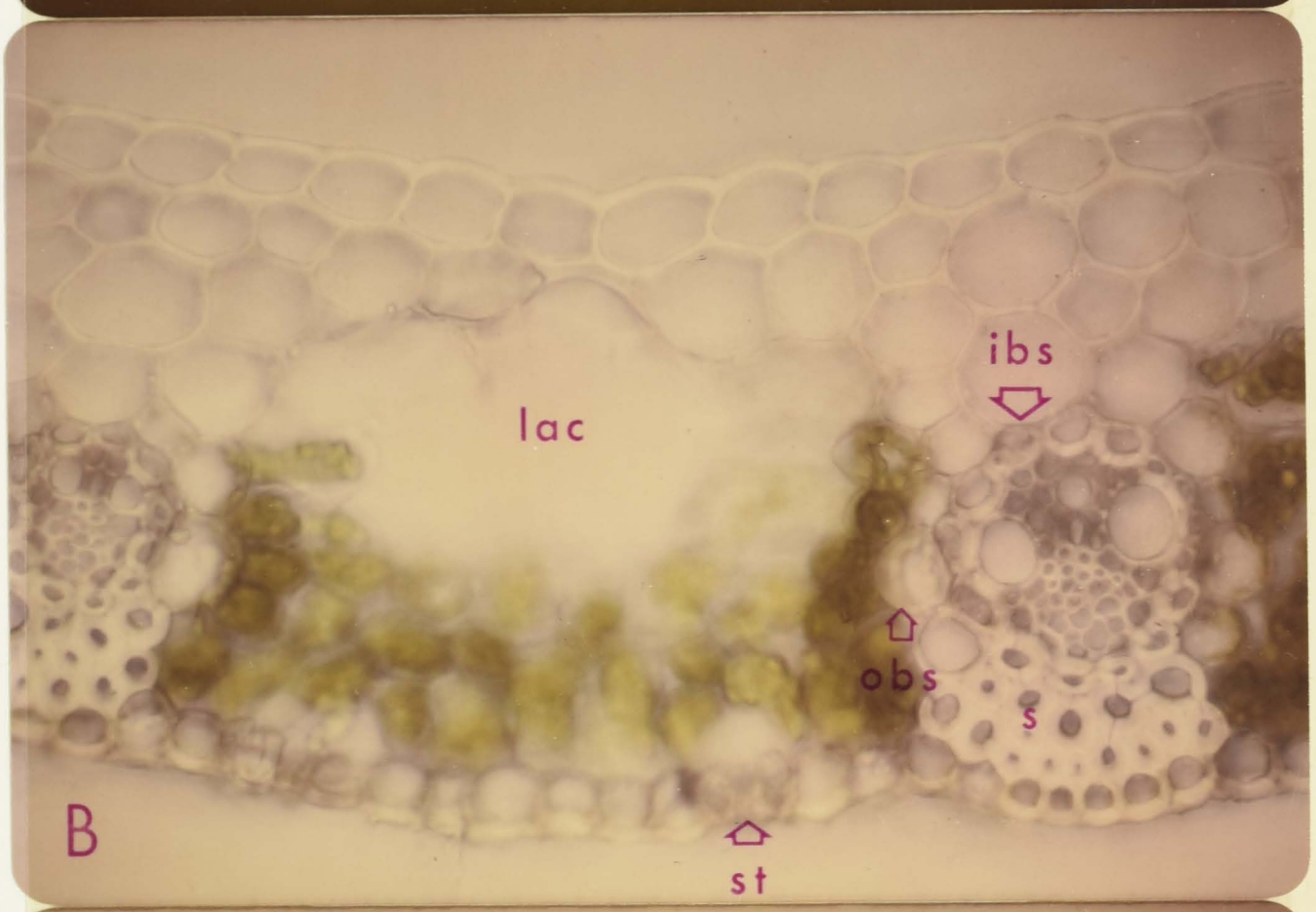
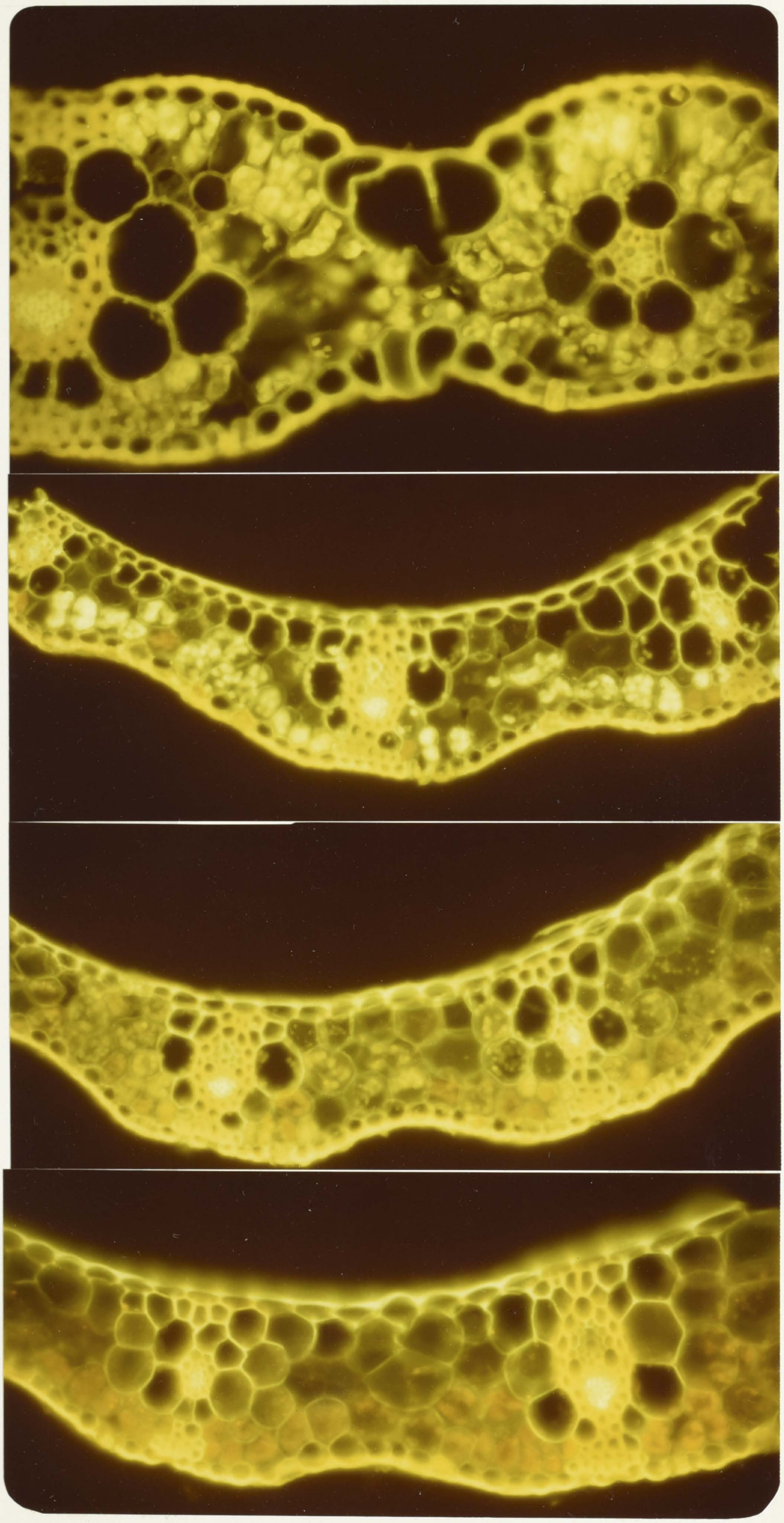


Fig. 19.

FIG. 19. Immunofluorescent labelling of RuP_2Case in hand transections of *Panicum pygmaeum* leaves (Gramineae, eu-panicoid, C_3, XyMS+); adaxial epidermis uppermost; prepared as in Chapter 5.3.5.. *A*, blade, anti-wheat RuP_2Case test (A/S-1), to show chloroplasts of all chlorenchymatous cells immunofluoresce; X410. *B*, sheath, anti-wheat RuP_2Case test (A/S-2), to show chloroplasts of all chlorenchymatous cells immunofluoresce as in the blade; X255. *C*, sheath, normal serum control; X255. *D*, sheath, autofluorescence control. X255. In sheath, note: chlorenchyma is abaxially situated, continuous between adjacent vascular bundles, and tends to be of constant depth (1 to 2 cells) from abaxial epidermis; lateral cell count >4.



A

B

C

D

Fig.19

Fig. 20.

FIG. 20. Immunofluorescent labelling of RuP_2Case in hand transections of *Panicum bulbosum* leaves (Gramineae, eu-panicoid, C_4, XyMS-); adaxial epidermis uppermost; prepared as in Chapter 5.3.5.. *A*, blade, autofluorescence control, showing blade anatomy, cf. sheath; X255. *B*, sheath, anti-wheat RuP_2Case test (A/S-2), to show clear immunofluorescent labelling response in PCR sheath cells only; X255. *C*, sheath, normal serum control; note stoma in abaxial epidermis; X410. *D*, sheath, autofluorescence control; X255.

In sheath, note: anatomy is nearly "classical", but many adaxial and interveinal "colourless cells" are present cf. blade above; "colourless cells" extend to abaxial epidermis, and have thicker walls than PCA cells; lateral cell count is ≤4 if "colourless cells" are ignored in the count; chloroplast-containing PCA and PCR cells extend to similar depths adaxially; XyMS condition as in blade; PCR sheath retains identity adaxially, but contains no chloroplasts there in large vascular bundles; sclerenchyma predominantly abaxial to vascular tissue.

cc, colourless cells; *pca*, PCA cells; *pcr*, PCR cells; *s*, sclerenchyma.

Fig. 20

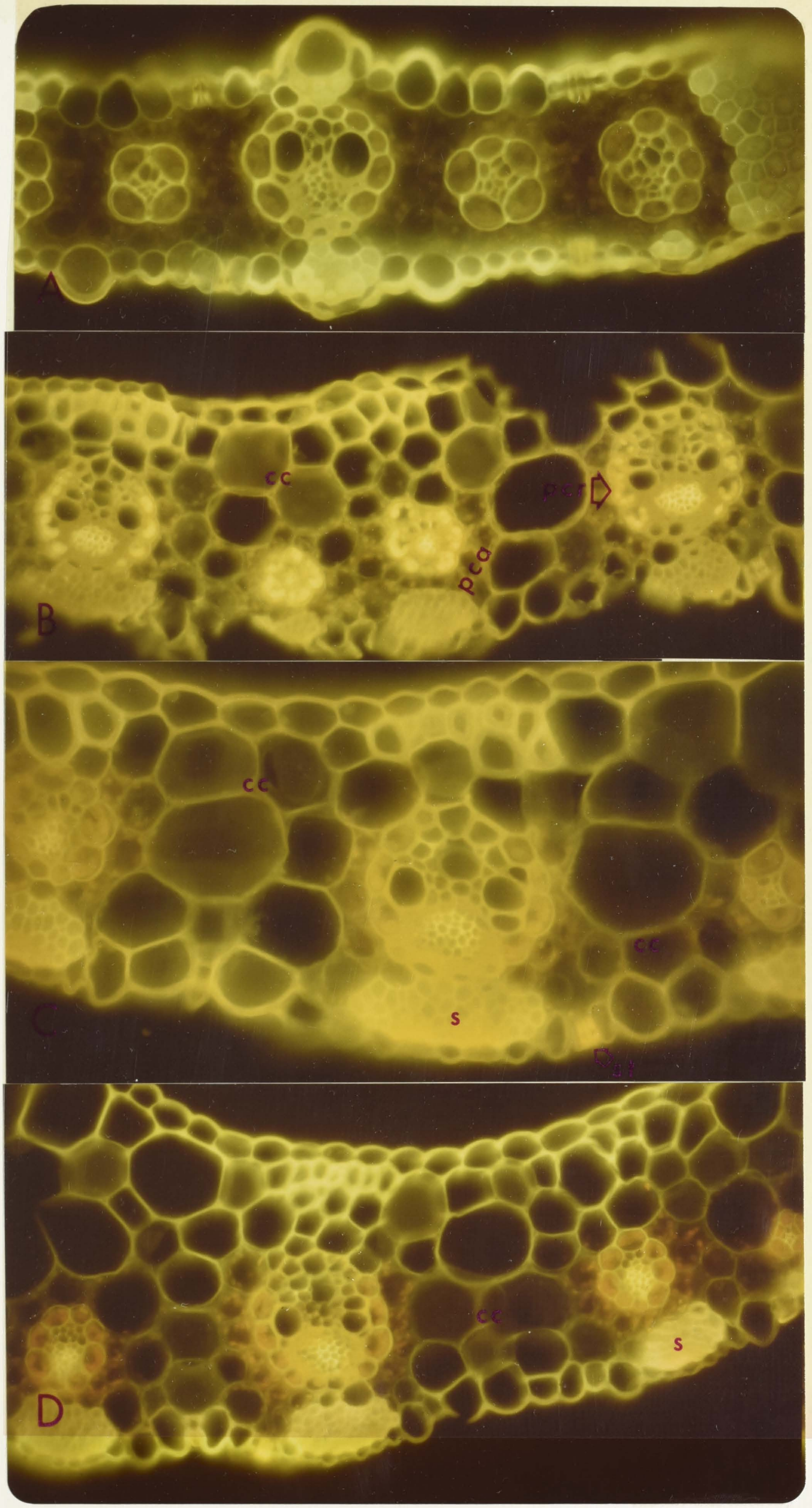
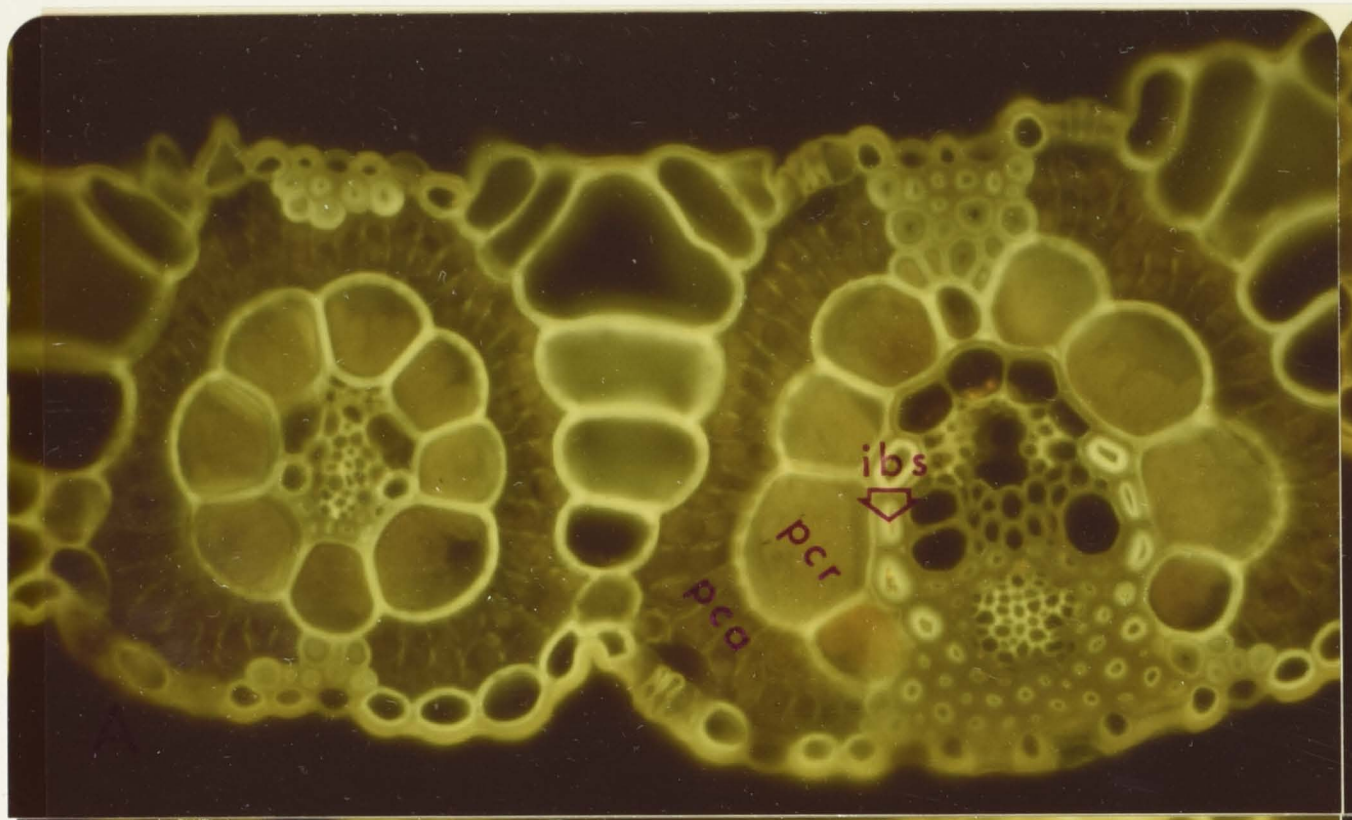


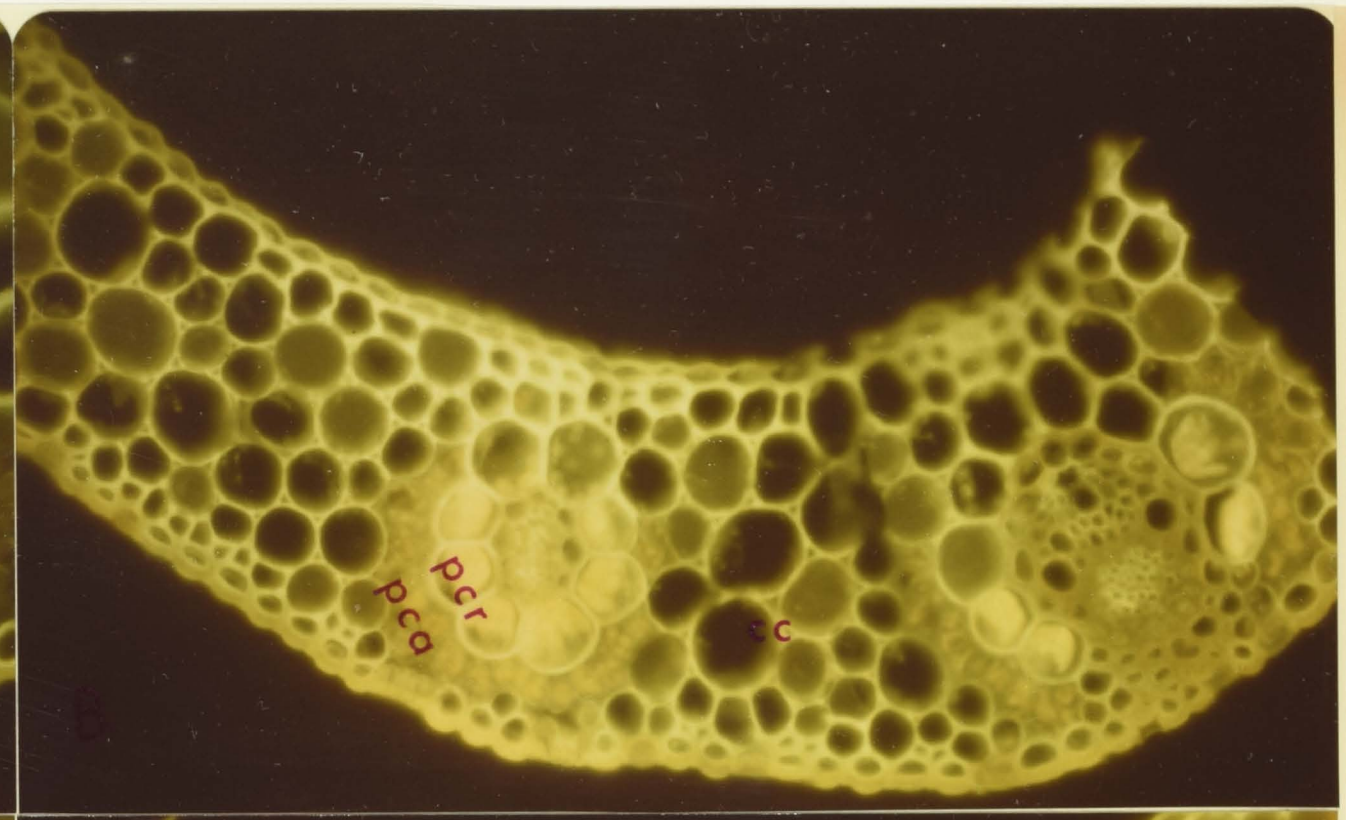
FIG. 21. Immunofluorescent labelling of RuP_2Case in hand transections of *Buchloë dactyloides* leaves (Gramineae, chloridoid, C_4, XyMS+); adaxial epidermis uppermost; prepared as in Chapter 5.3.5..

A, blade, normal serum control, to show blade anatomy cf. sheath; PCA cells radiate about PCR sheath cells; PCR cells larger in transectional area than PCA cells; small bundle has one recognisable sheath cf. large bundle with two; X255. *B*, sheath, anti-wheat RuP_2Case test (A/S-2), to show clear immunofluorescent labelling response in PCR sheath cells only; X255. *C*, sheath, normal serum control; X410. *D*, sheath, autofluorescence control; X255.

In sheath, note: anatomy is nearly "classical", but many adaxial and interveinal "colourless cells" are present cf. blade above; "colourless cells" extend to abaxial epidermis, and have thicker walls than PCA cells; lateral cell count is <4, ignoring "colourless cells"; chlorenchyma absent adaxially, and extending to a constant depth from abaxial epidermis; chloroplast-containing PCA and PCR cells extend to similar depths adaxially; XyMS condition as in blade; sheath in "mestome sheath" position (inner sheath) retains identity adaxially (for large bundles only).

cc, colourless cells; *pca*, PCA cells; *pcr*, PCR cells; *ibs*, inner bundle sheath.





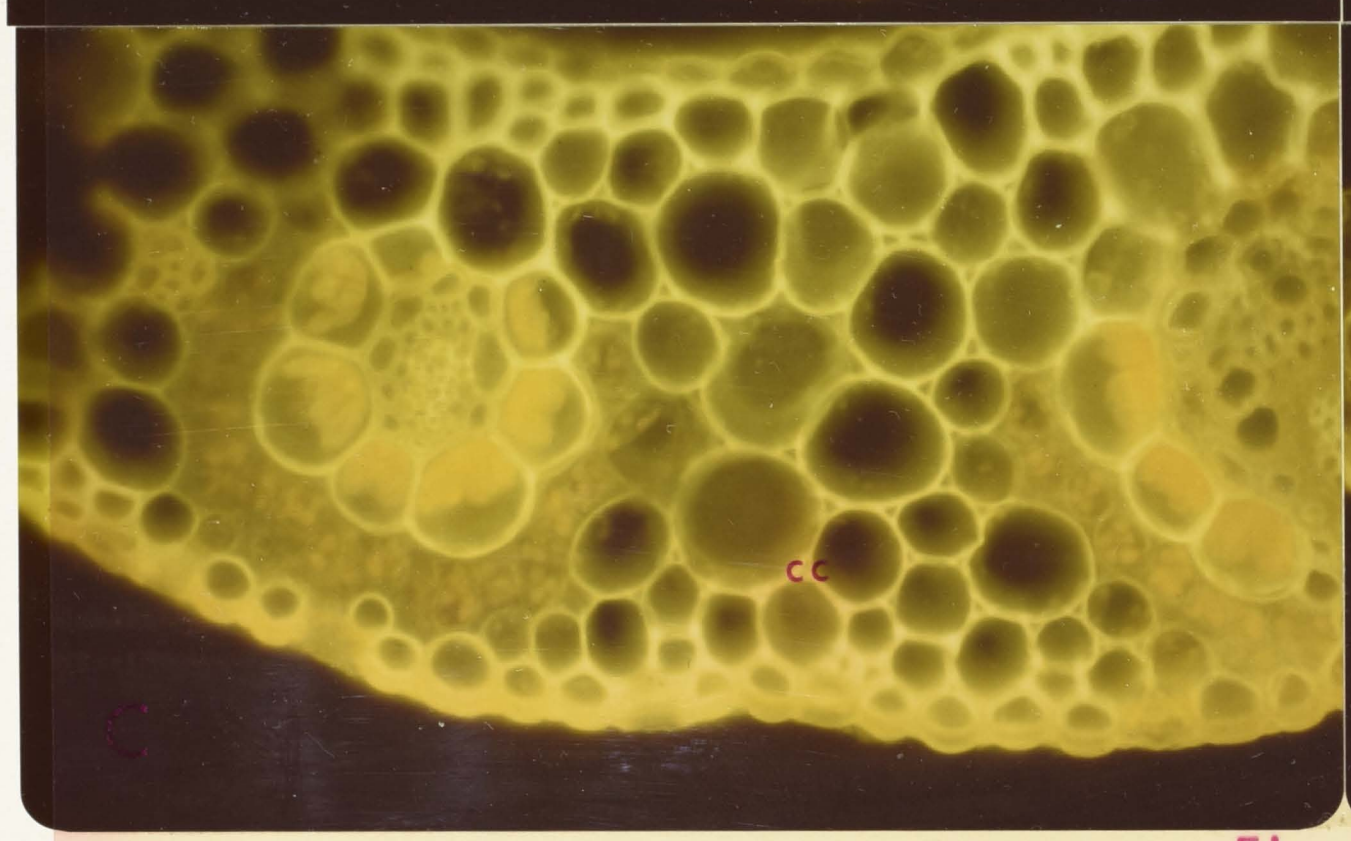


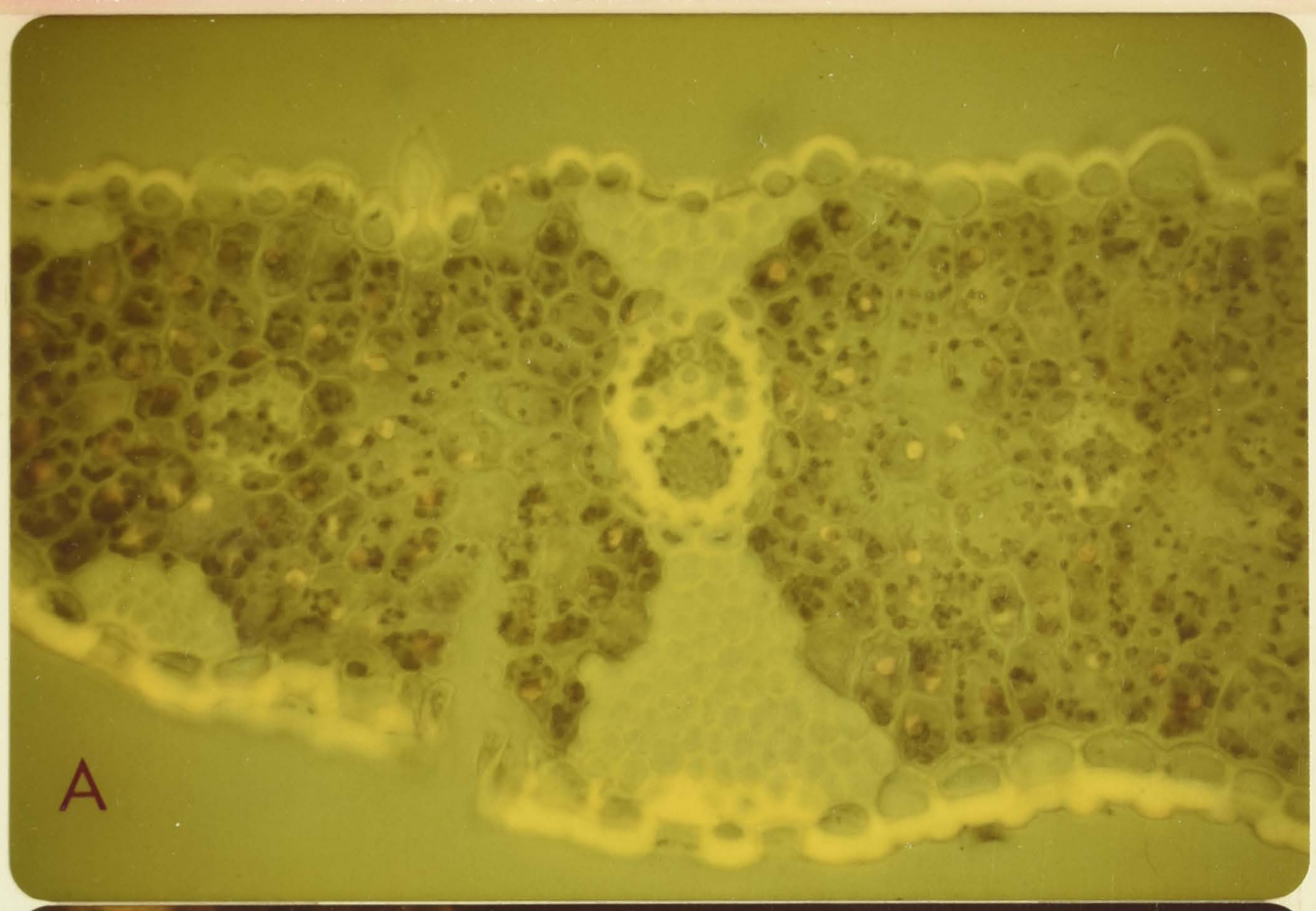
Fig. 21

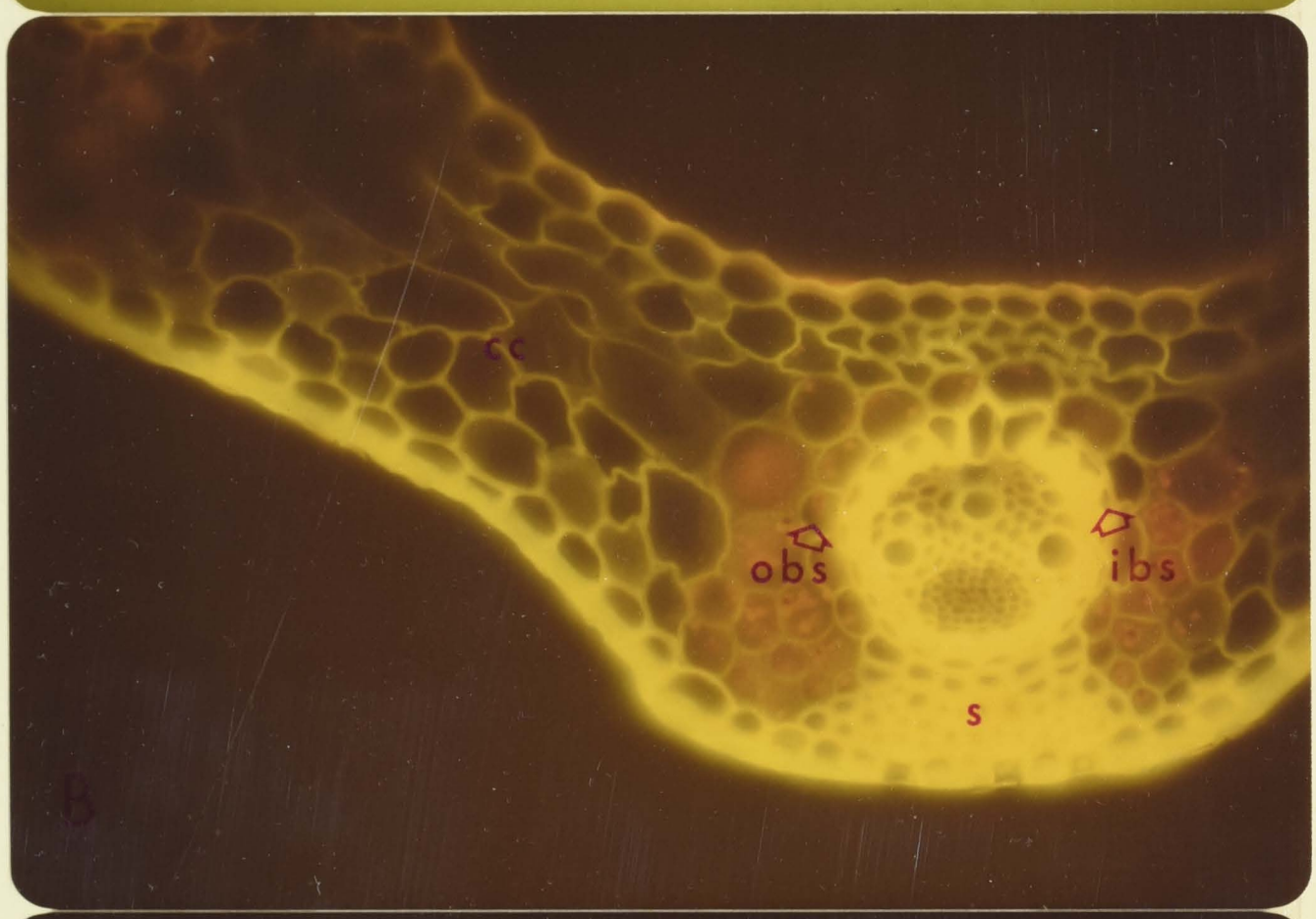
Fig. 22.

FIG. 22. Transections of grass leaves; adaxial epidermis uppermost. *A*, *Danthonia carphoides* (danthonioid,C_3), blade, to compare with sheath below; lateral cell count is low (3-4) between vascular bundles shown, but "one cell distant criterion" is frequently violated; no "colourless cells", cf. sheath below; prepared as in Chapter 6.3., observed in epifluorescence + brightfield; X410. *B*, *Danthonia carphoides* (danthonioid, C_3), sheath, to show atypical C_3 leaf sheath anatomy; "colourless cells" are in contact with abaxial epidermis as is usual in C_4 leaf sheaths; "one cell distant criterion" violated, but not often; outer bundle sheath contains chloroplasts; inner sheath retains integrity adaxially; sclerenchyma associated with vascular tissue abaxially; prepared as in Chapter 6.3., observed in epifluorescence; X410. *C*, *Panicum milioides* (eu-panicoid, putative C_3/C_4 intermediate), sheath; to compare with blade (Fig. 16C); exhibits C_3 leaf sheath anatomy, with chlorenchyma continuous between vascular bundles (cf. Figs. 19D); chlorenchyma abaxially situated, one or two cells deep; sclerenchyma associated with vascular tissue abaxially; epifluorescence micrograph, prepared as for autofluorescence controls Chapter 5.3.5.; X410.

cc, colourless cells; *obs*, outer bundle sheath; *ibs*, inner bundle sheath; *s*, sclerenchyma.

Fig. 22





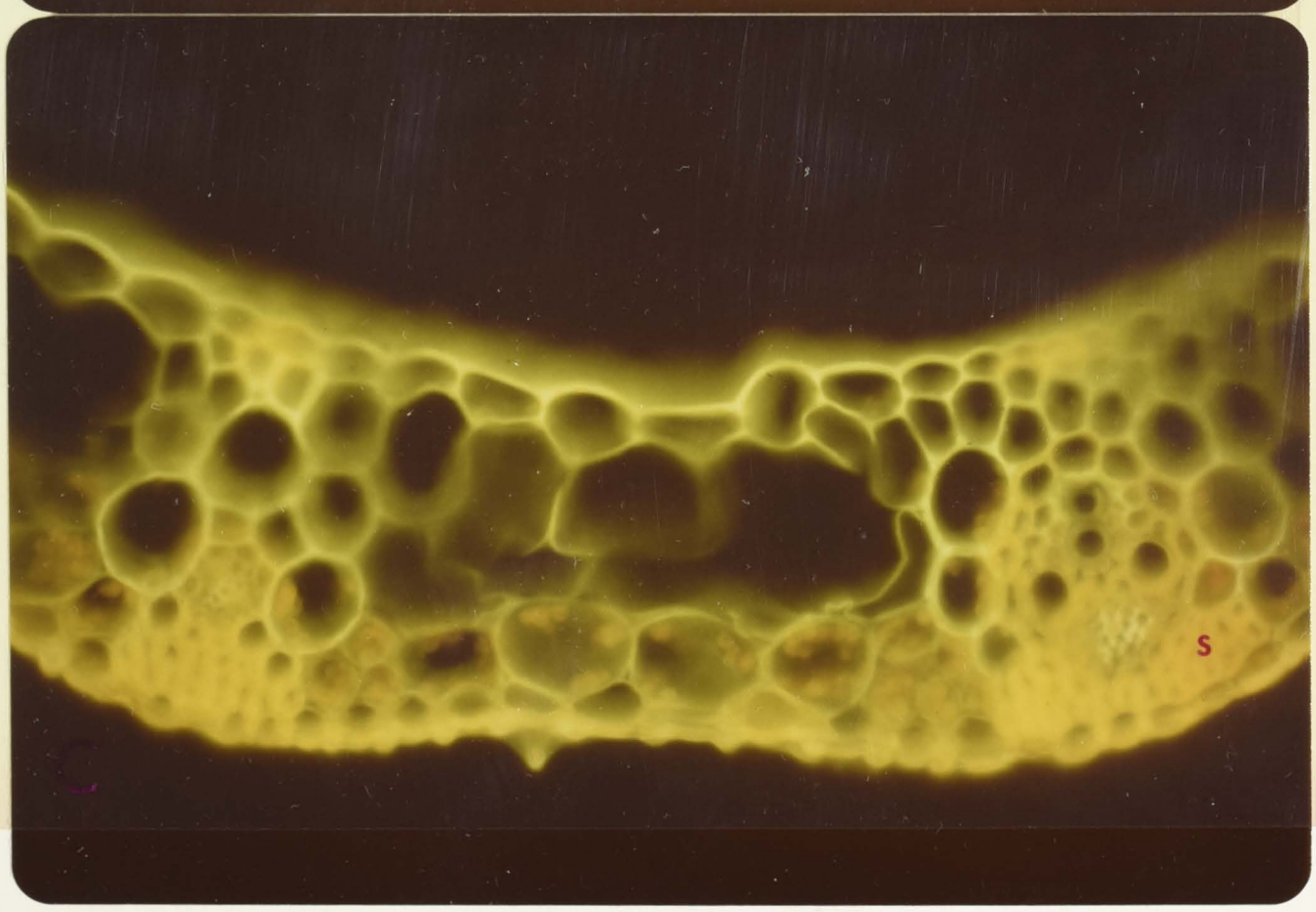
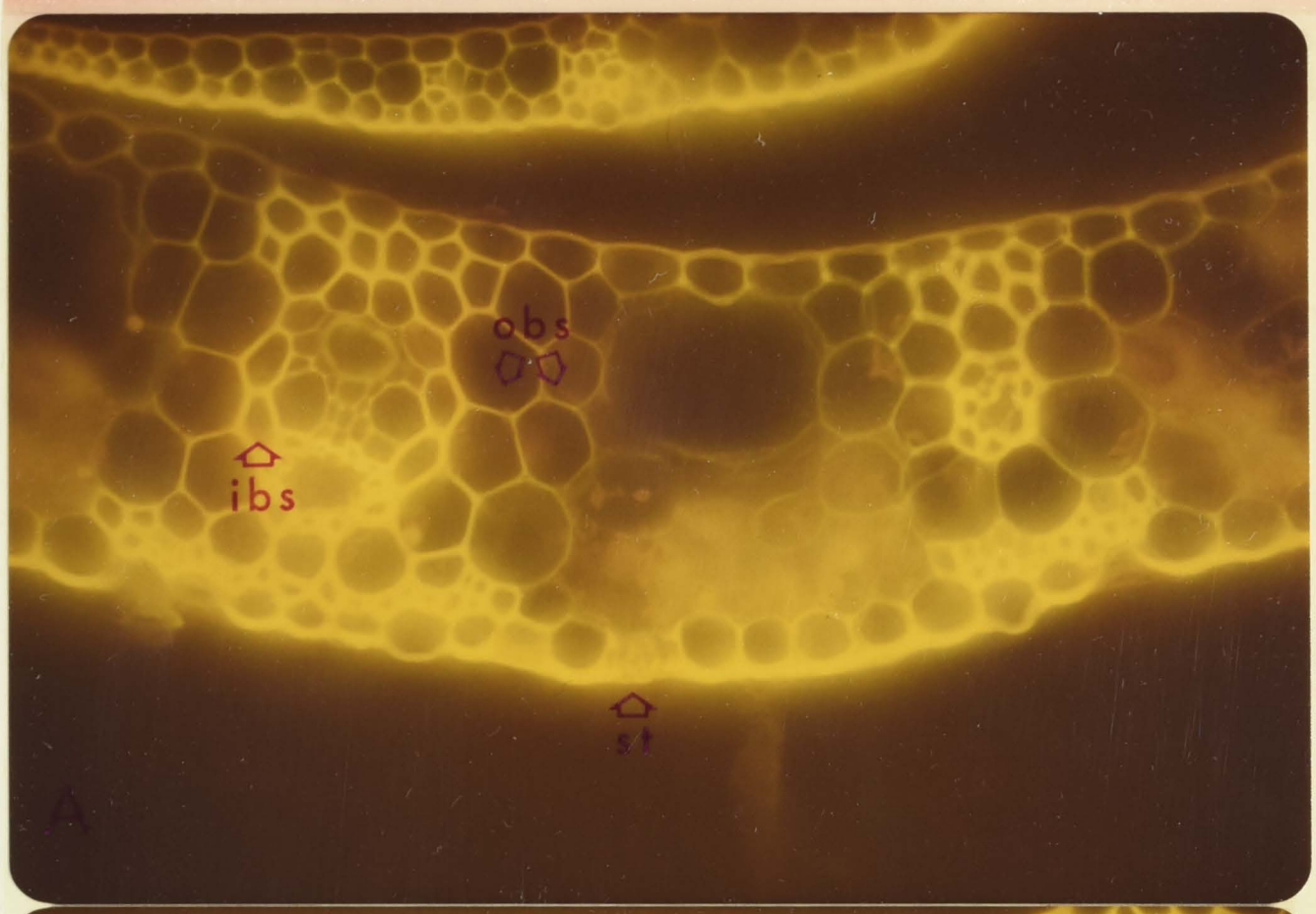


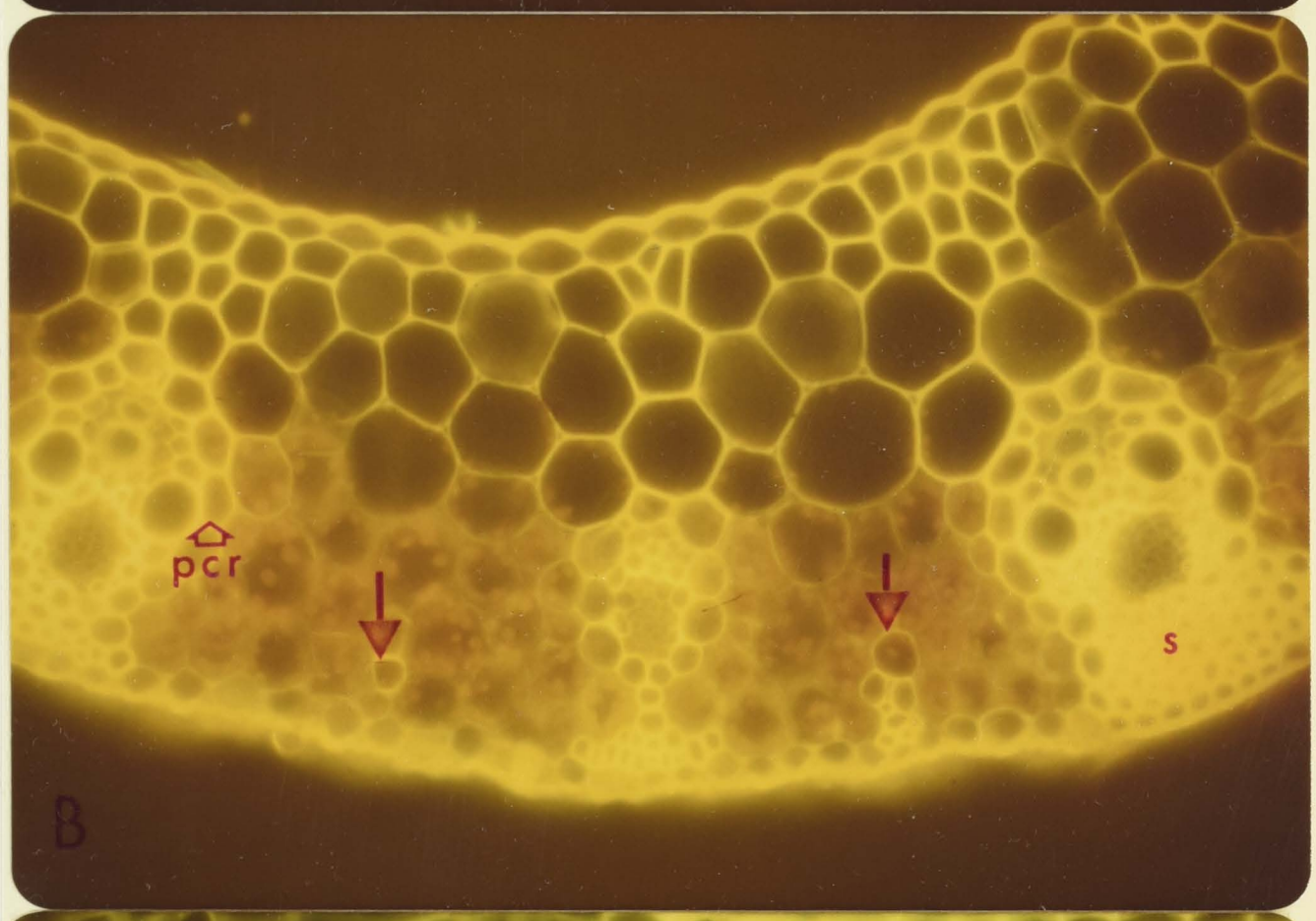
Fig. 23.

FIG. 23. Transections of grass leaf sheaths; abaxial epidermis at bottom; all epifluorescence micrographs.
A, *Isachne globosa* (eu-panicoid, C_3), "Isachne-type" C_3 leaf anatomy not manifested (cf. Figs. 2C, 3A for blades of other species); outer (parenchyma) bundle sheath may be double in places (arrowed); note stoma in abaxial epidermis; adaxially, inner sheath cells tend to merge with other tissue; prepared as in Chapter 6.3.; X410. *B*, *Arundinella nepalensis* (arundinoid, C_4), "Arundinella type" C_4 leaf anatomy not clearly manifested, isolated PCR strand cells (arrowed) less conspicuous (cf. blade, Fig. 8); "one cell distant criterion" holds if strand cells considered; strand cells appear to be associated with some vascular tissue; chlorenchyma abaxially situated and of constant depth; sclerenchyma associated with vascular tissue abaxially; PCR sheath remains discrete adaxially; XyMS condition as in blade; prepared as in Chapter 6.3.; X255. *C*, *Alloteropsis semialata* (eu-panicoid, C_4), to be compared with blade (Fig. 12C); PCR still inner of two recognisable sheaths, but its cells smaller in transectional area than those of outer bundle sheath; PCR cells contain chlorophyll adaxially; abaxially PCR sheath cells nearly occluded; PCR sheath remains discrete; XyMS condition as in blade; note torn cell walls in lacuna; note "pair" of vascular bundles (arrowed), cf. Fig. 25A; prepared as autofluorescence control, Chapter 5.3.5.; X325.

st, stoma; *s*, sclerenchyma; *lac*, lacuna; *pcr*, PCR tissue; *ibs*, inner bundle sheath (=PCR); *obs*, outer bundle sheath.

Fig. 23





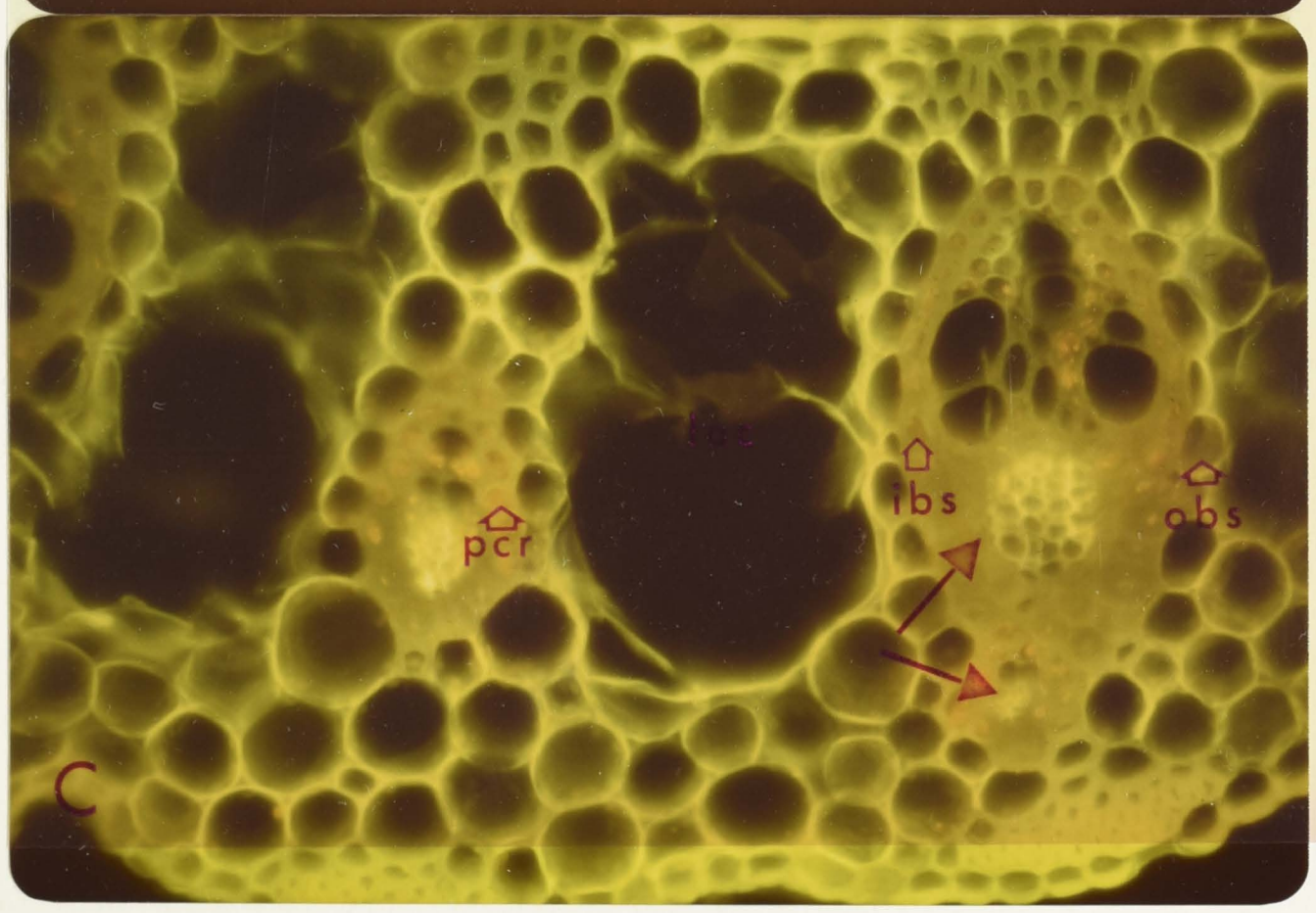
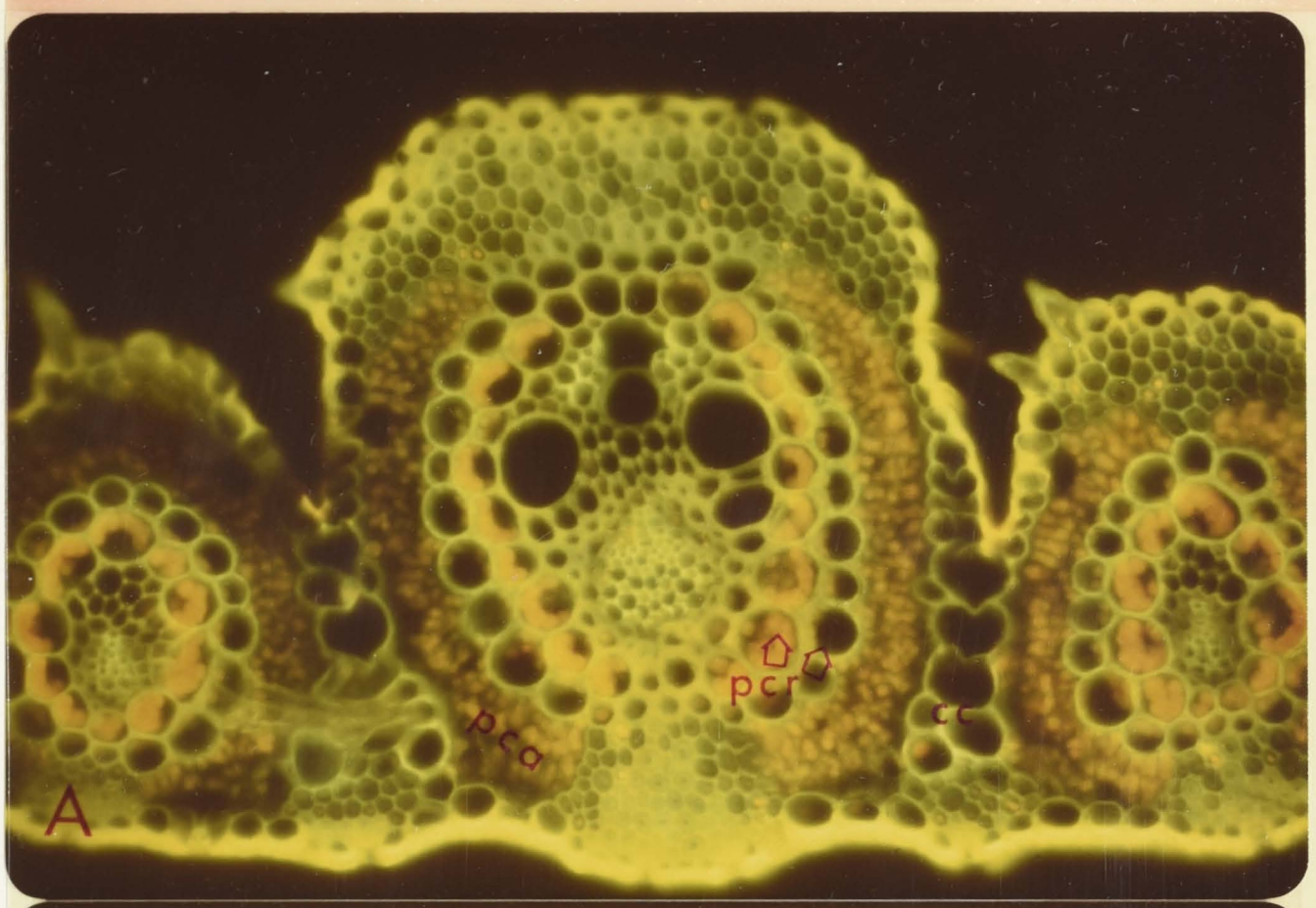


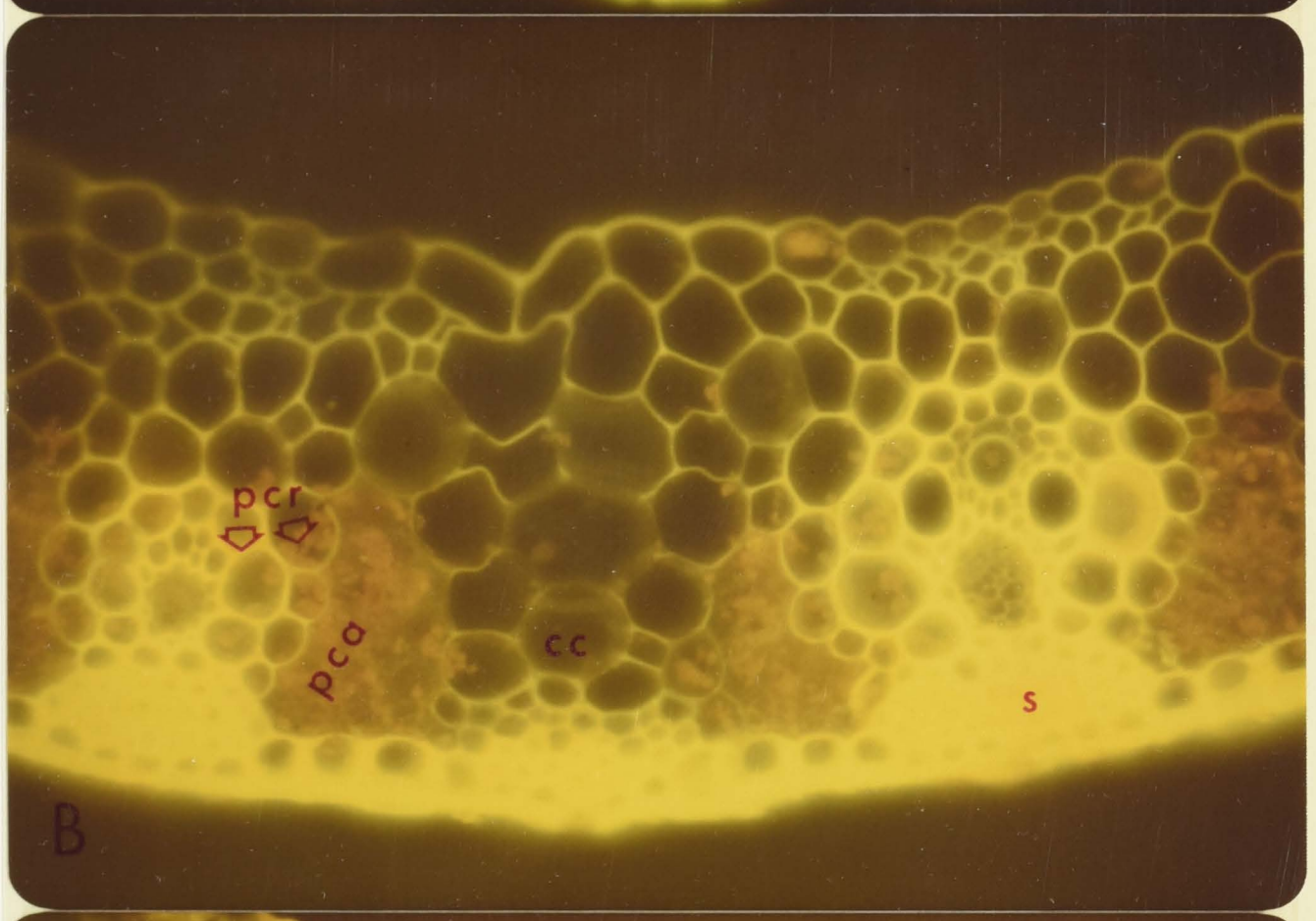
Fig. 24.

FIG. 24. Transections of grass leaves; adaxial epidermis uppermost; all epifluorescence micrographs. *A*, *Aristida biglandulosa* (Aristideae, C_4, XyMS-), blade; to compare with sheath below; note relative lack of "colourless cells" cf. sheath, and double PCR sheath (cf. Fig. 13A), outer with less dense chloroplasts; prepared as autofluorescence control, Chapter 5.3.5.; X255. *B*, *Aristida biglandulosa* (Aristideae, C_4, XyMS-), sheath; PCR double as in blade (above); "colourless cell" tissue is extensive, chlorenchyma abaxially situated; chloroplast-containing PCA and PCR cells extend to similar depths adaxially; sclerenchyma associated with vascular tissue abaxially; note lack of adaxial ribbing in leaf; prepared as in Chapter 6.3.; X325. *C*, *Triodia pungens* (danthonioid, C_4, XyMS+), sheath; to be compared with blade (Fig. 14B); PCR cells do not form "draping layers"; PCR sheaths abut onto abaxial epidermis; chlorenchyma abaxially situated; chloroplast-containing PCA and PCR cells extend to similar depths adaxially; "colourless cells" contain a few chloroplasts; abaxial ribbing more, and adaxial ribbing less prominent than in blade; prepared as in Chapter 6.3.; X165.

cc, colourless cells; *s*, sclerenchyma; *pca*, PCA cells; *pcr*, PCR cells.

Fig. 24





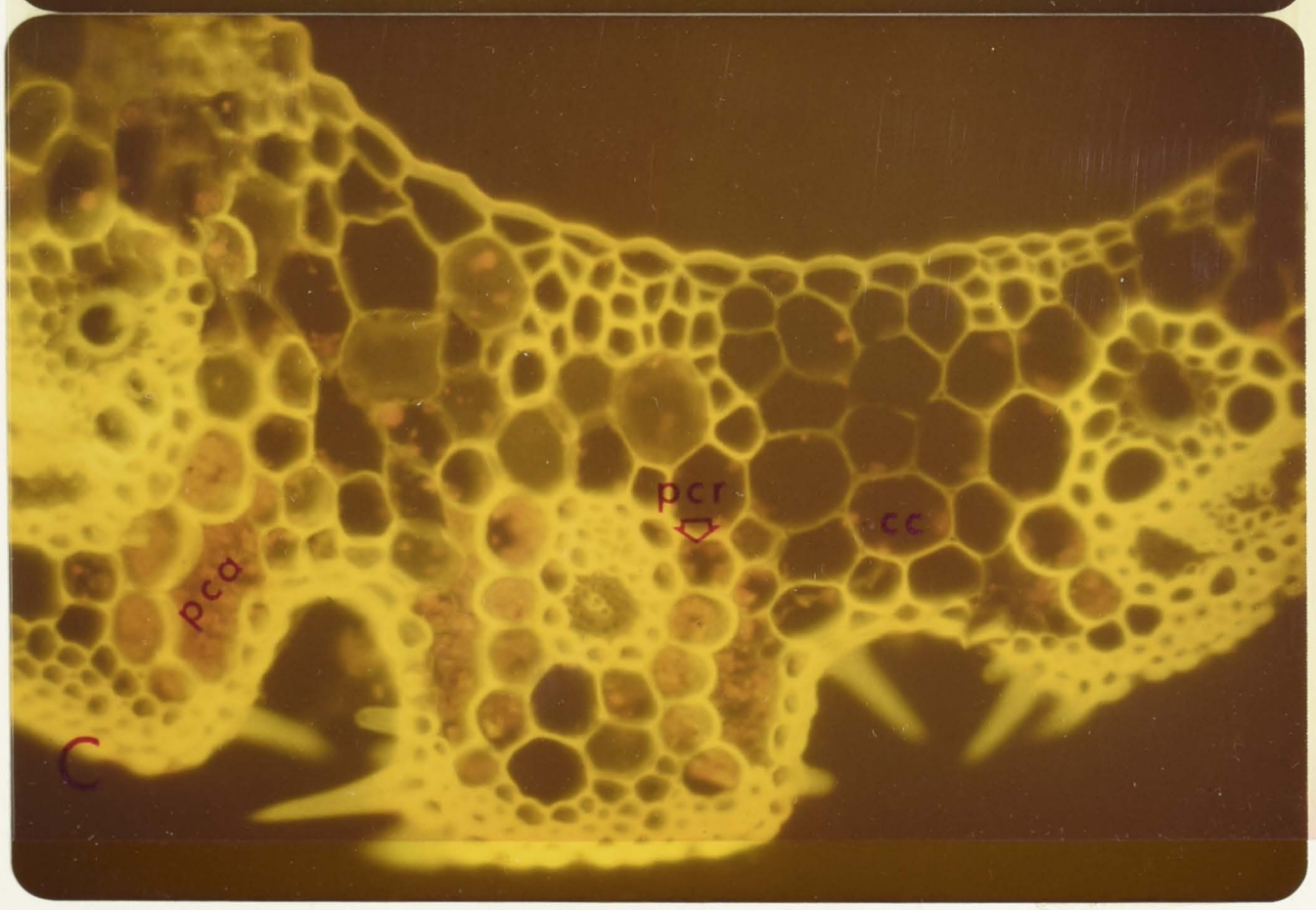


Fig. 25.

FIG. 25. Transections of grass leaf sheaths; adaxial epidermis at bottom (just out of frame in A); epifluorescence micrographs, prepared as in Chapter 6.3.. *A*, *Saccharum officinarum* (andropogonoid, C_4), to show vascular bundle "pairing"; PCR sheath of small vascular bundle abuts onto large vascular bundle; three bundle sheaths can be recognised around large vein at level of metaxylem vessel elements: outer (parenchyma?) bundle sheath, inner (mestome?) bundle sheath, and an extra layer within the latter (cf. Fig. 4A); XyMS condition cannot be assessed; "colourless cells" contain scattered chloroplasts; X165. *B*, *Chloris gayana* (chloridoid, C_4); chlorenchyma abaxially situated; chloroplast-containing PCA and PCR cells extend to similar depths adaxially; "colourless cells" extend to abaxial epidermis; PCR sheath appears double in places (arrowed); X255.

cc, colourless cell; *mx*, metaxylem vessel element; *pca*, PCA cells; *pcr*, PCR cells; *obs*, outer bundle sheath; *ibs*, inner bundle sheath; *xl*, extra layer.

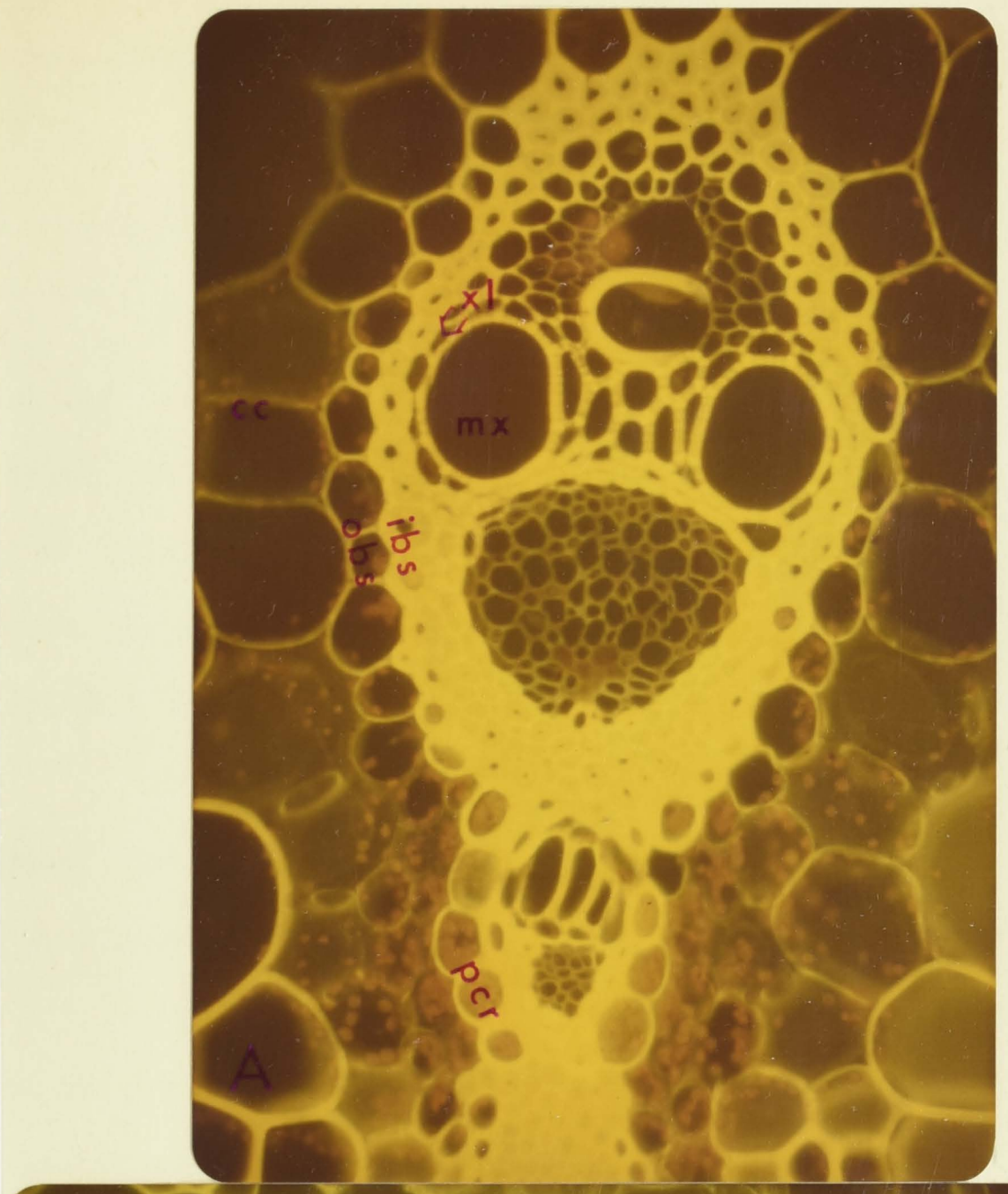
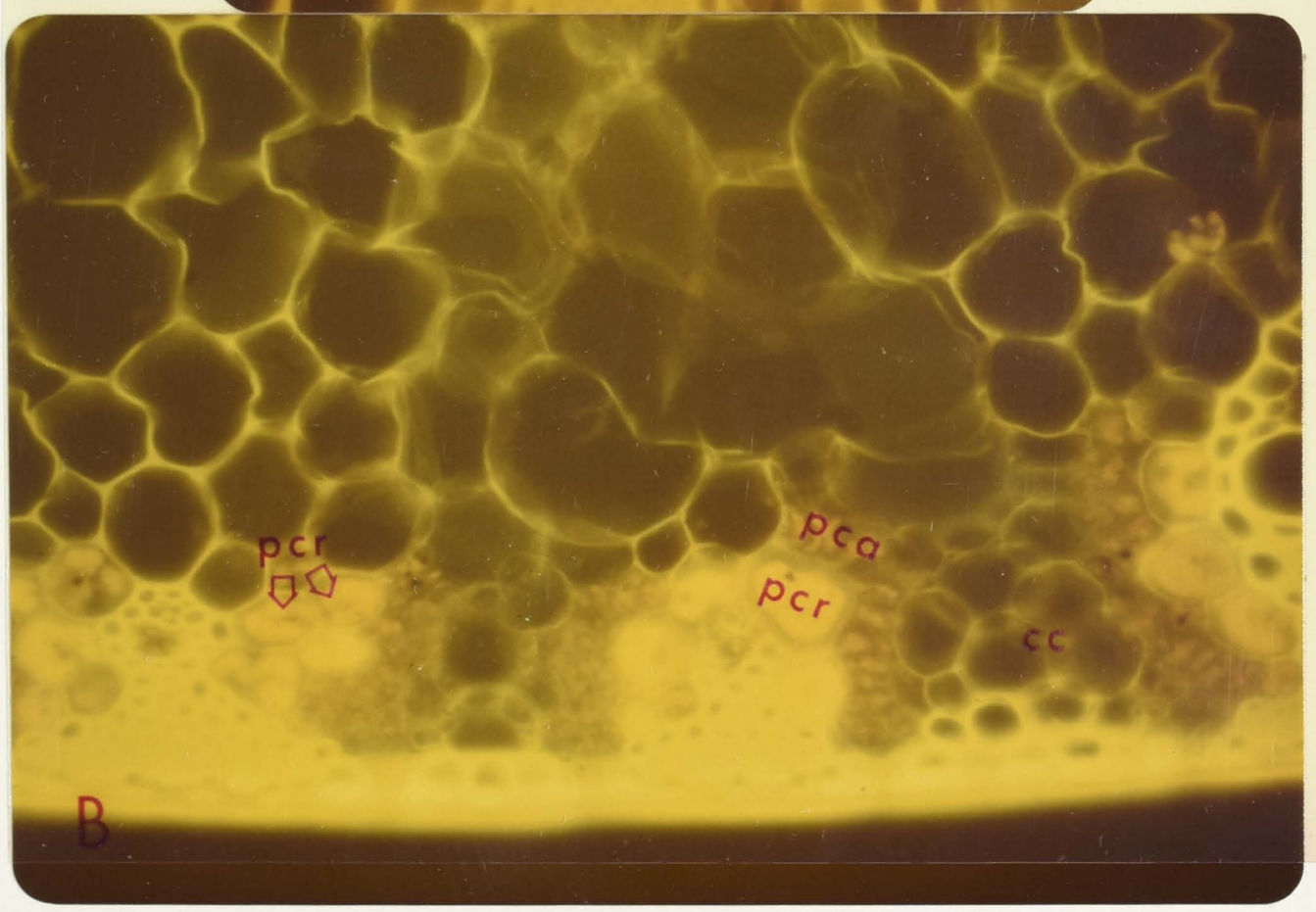


Fig. 25



A P P E N D I X

This Appendix outlines areas for future work, which supplement those given throughout the text.

A.1 GENERAL AREAS

(i) Although I have implied that the *Arundinella* type of leaf anatomy is a distinct C_4 anatomical variant, examination of Tateoka's (1958, 1963) reports on leaf anatomy in *Arundinella* and *Garnotia* reveals an interesting range of variants on the "isolated PCR strand" theme. These variants may be especially useful in assessing the functional significance of different PCA-PCR relationships.

(ii) What biochemical types are found in C_4 species of the Cyperaceae (cf. Chen *et al.* 1974), and in other families, and how do they compare with those in the Gramineae?

(iii) What are the taxonomic relationships between the C_3 and C_4 eu-panicoid species? Watson (1976 - unpublished) has produced preliminary classifications of eu-panicoid grasses employing the information gain computer program 'MULTBET' (Lance and Williams 1967) and the data provided in Clifford and Watson (1976). Even when the attributes "photosynthetic pathway" (C_3 or C_4) and the "XyMS condition" (XyMS+ or XyMS-) were included in the analyses, major groups generated contained mixtures of both C_3 and C_4 species and both XyMS+ and XyMS- species. Subgroups at lower levels are sometimes inconsistent for these two attributes also. For example, the *Panicum* spp. (*P. antidotale*, C_4, XyMS-; *P. bulbosum*, C_4, XyMS-; *P. effusum*, C_4, XyMS+; *P. maximum*, C_4, XyMS+; *P. milioides*, C_3/C_4, XyMS+; *P. pygmaeum*, C_3, XyMS+; *P. trichoides*, C_3, XyMS+) remain in one group.

A.2 SOME FURTHER APPLICATIONS OF *IN SITU* IMMUNOFLUORESCENT LABELLING

(1) *Of ribulose-1,5-bisphosphate carboxylase*

(a) To test if RuP_2Case is present in guard cell chloroplasts (Raschke 1976; cf. Allaway 1976).

(b) To investigate the claim that *Sorghum* changes photosynthetic pathway at anthesis (Khanna and Sinha 1973).

(c) Leaf transections of many dicotyledonous species illustrated by Rathnam *et al.* (1976) appear to violate the "one cell distant criterion" described in Chapter 2 for grasses. Labelling of RuP_2Case in these leaves would show to which cells the enzyme was compartmented.

(d) Kagan-Zur and Lips (1975) have suggested that RuP_2Case may be located in microbodies in the dark, and that in the light, microbodies fuse with chloroplasts. Dark pre-treated leaves of the species they used for their work, need to be subjected to RuP_2Case labelling.

(e) Huber *et al.* (1976) have shown that Fraction I protein is absent from differentiated PCA cells of C_4 plant leaves i.e., lack of activity of RuP_2Case is not due to *in*activation of RuP_2Case. My immunofluorescent labelling results (Chapter 5) confirm this conclusion. However, the question still remains as to whether RuP_2Case is absent from PCA cells in all leaf developmental stages.

(2) *Of other photosynthetic enzymes*

(a) To locate PEP carboxylase. Is it cytoplasmic, as most workers suggest (e.g., Hatch and Osmond 1976), or chloroplastic?

(b) To locate PEP carboxykinase, and aspartate and alanine aminotransferases in PCK type C_4 species (see Hatch and Osmond 1976). Rathnam and Edwards (1975) have concluded that PEP

carboxykinase is chloroplastic, aspartate aminotransferase is mitochondrial, and alanine aminotransferase is cytoplasmic in *Panicum maximum*.

I made some attempts to locate pyruvate Pi dikinase in C_4 and C_3 plant leaves by immunofluorescent labelling using antisera kindly supplied by Drs. T. Akazawa and T. Sugiyama. Use of anti-pyruvate Pi dikinase serum should have furnished a means of identifying PCA cells in C_4 species, and could have been very interesting for application to *Panicum milioides*. Pyruvate Pi dikinase is the only enzyme held to be unique to C_4 plant leaves (in PCA cells: see Hatch and Osmond 1976).

Anti-maize pyruvate Pi dikinase serum (γ-globulin) (prepared according to Sugiyama and Laetsch 1975) was applied to leaf transections of *Zea mays* (C_4), *Pennisetum villosum* (C_4), *Buchloë dactyloides*(C_4), *Panicum milioides* (intermediate?), and *Microlaena stipoides* (C_3) (following the method in Chapter 5.3.5.). *All* chloroplasts of *all* chlorenchymatous cells in *all* species immunofluoresced. Appropriate controls and RuP_2Case tests were run concurrently, and gave expected results (see Chapter 5).

The anti-pyruvate Pi dikinase results are in conflict with all that is known on the location of this enzyme from other studies (see Hatch and Osmond 1976). Perhaps the antiserum contained heterologous antigens, and was not specific for pyruvate Pi dikinase.

A.3 *PANICUM MILIOIDES*

In Chapter 5.4.2.D., it was suggested that in *Panicum milioides* leaves there may be a *limited* linked PEP-carboxylation - C_4 acid decarboxylation cycle (C_4 cycle) *between* "mesophyll" and "bundle sheath" (BS) cells. In normal air, flux of carbon through C_4 acids, to the Calvin Cycle in BS cells, would appear to be insignificant in this species (e.g.,

see Kanai and Kashiwagi's (1975) kinetic ^{14}C labelling results); therefore, if a limited C_4 cycle *is* present, rates of carbon assimilation via PEP carboxylase must be very low in contrast to that via RuP_2Case. The limiting factor on C_4 cycle activity is, in my hypothesis, the rate of regeneration of PEP. If the C_4 acid decarboxylase system in *P. milioides* is restricted to BS cells, one could suggest on the basis of anatomical observations, that this system has insufficient capacity to cope with potential rates of C_4 acid production in the mesophyll. The chloroplasts in BS cells of *P. milioides* are, superficially, less dense than in C_4 plants, so that although the mesophyll/bundle sheath volume ratio falls within the C_4 range (Chapter 7.2), one could envisage an imbalance of typical PCA to PCR tissue activity. Furthermore, the maximum lateral cell count lies within the C_3 range (Chapter 2.4).

Insufficient PEP regeneration, and therefore inadequate supply to mesophyll cells, would mean that RuP_2Case in mesophyll cells (whose presence was demonstrated by immunofluorescent labelling: Chapter 5.4.2.D.) would compete with PEPCase for carbon from the CO_2 - HCO_3^- equilibrium system such that most carbon is primarily fixed via RuP_2Case. Under conditions of low intercellular CO_2 concentrations (e.g., near the CO_2 compensation point of *P. milioides*), the proportion of carbon flux into C_4 acids in mesophyll cells compared with flux directly into 3-phosphoglyceric acid (3-PGA), may be higher than in normal air conditions. The critical work needed to test my hypothesis seems, therefore, to be a series of ^{14}C radiotracer pulse-chase experiments *near* the CO_2 compensation point, to assess whether a significant amount of label is transferred from C_4 acids to 3-PGA under these conditions.

At the CO_2 compensation point of *P. milioides*, intercellular CO_2 concentration in the mesophyll would be about 20 ppm. (i.e., the measured CO_2 compensation point of *P. milioides* leaves). Since mesophyll cells contain RuP_2Case, and presumably a complete Calvin Cycle and glycolate

pathway, photorespiratory CO_2 production via RuP_2-oxygenation would exceed photosynthetic CO_2 assimilation via RuP_2 carboxylation i.e., these cells are not at their "CO_2 compensation point" with respect to the RuP_2 carboxylase-oxygenase system. The difference in rates of photorespiratory CO_2 production and photosynthetic CO_2 assimilation (via RuP_2Case) in mesophyll cells would equal the rate of PEP carboxylation in mesophyll cells, and this in turn would equal rates of *net* carbon assimilation in the bundle sheath cells. In other words, at the CO_2 compensation point in *P. milioides*, there may be a net carbon flow from mesophyll to bundle sheath cells (a previously unknown phenomenom), and it would be interesting to speculate on the significance of this "biochemical carbon pump" in relation to carbon transport relative to rates of diffusion of, say, sucrose.

The CO_2 compensation point value in *P. milioides* would be determined by the *relative rates* of PEP carboxylation, RuP_2 carboxylation, and RuP_2 oxygenation *in mesophyll cells;* PEP carboxylase being part of a C_4 cycle which is cellularly compartmented as in C_4 plant leaves. The rate of PEP carboxylation may only need to be very low to effect a reduction in CO_2 compensation point from typical C_3 plant values to 20 ppm.. For this reason, substantiation of my hypothesis may be difficult.

P. milioides may, therefore, be a genuine C_3/C_4 intermediate. If so, it is intriguing that a species has been found where the *C_4 cycle is compartmentalised*, an anatomical stoichiometry has not yet been achieved, and a complete Calvin Cycle is not yet restricted to bundle sheath cells. If PCR cell capacity did match PCA cell capacity in *P. milioides* leaves, would its photosynthetic physiology be distinguishable from that of C_4 plants even if a Calvin Cycle were still present in mesophyll cells? I have wondered whether slight PCA cell chloroplast immunofluorescence in some C_4 plant leaves (Chapter 5.4.2.B.) is of any

significance in this context. Perhaps it is necessary to temper the natural tendency to rigidly compartment biological systems and processes in order to understand them.